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CONTENTS.

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YELLOW FEVER RESEARCH, 1918–1924: A SUMMARY.

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Isolation of Leptospira icteroides.

Leptospira icteroides was first isolated in Guayaquil, Ecuador, in 1918 (1), from six of twenty-seven selected cases of yellow fever diagnosed by Pareja and Elliott (2). The same organism was isolated by Kligler and myself (3) in Mérida, Mexico, in 1919–1920, from blood taken from a yellow fever patient on two successive days, the second and third of his illness. Pérez Grovas (4) obtained a strain of *L. icteroides* in Vera Cruz in 1920 from one of two cases of yellow fever studied, and in the same year Kligler and I (5) isolated the organism from four of thirteen cases in northern Peru. Le Blanc (6) succeeded in isolating a strain from one of two early cases in Vera Cruz in 1921. The latest positive results (1923–24) have been obtained in Palmeiras, Bahia, Brazil, by Vianna and Bião, of the American-Brazilian Yellow Fever Commission, who isolated two strains from two of nine cases (7). Negative results were obtained by Lebredo (8) in Mérida, Mexico, in the summer of 1919, and by Borges Vieira (9) in the suburbs of Bahia in 1920.

One of the Guayaquil patients died four days after the onset of illness, and the liver, which was removed a few hours post mortem, was found to contain a small number of leptospiras; guinea-pigs were inoculated with the suspension of the liver, and a strain of *L. icteroides* was obtained from the passage animals.

Of the sixteen positive blood specimens,[1] twelve were withdrawn between one and three days, two between three and four days, and two a little later than four days after onset; hence the indications are that the organism is present in the peripheral circulation more frequently (88 per cent of cases) during the first three days of the disease.

The total number of strains of *L. icteroides* isolated is therefore fifteen.

[1] One of the fifteen patients yielded positive results in the case of specimens drawn on two successive days.

TABLE I.

Isolation of Leptospira icteroides.

	Day of illness when blood was withdrawn	Results of			
		Dark-field examination	Direct inoculation into guinea-pigs	Direct cultivation	Cultivation from passage guinea-pigs
Guayaquil (Noguchi)					
1. Case 1 (died)	4 days and a fraction		Blood +	+	+
2. " 2 (recovered)	3 " " "		Blood +		−
3. " 3	2 " " "		Blood +		++
4. " 4 (died)	2 " "	Liver +	{ Blood +, Liver +	+	++
5. " 5	4 " "		Blood +		++
6. " 6	3 " "		Blood +		+
Mérida (Noguchi and Kligler)					
7. Case 2 (recovered)	1 day and a fraction		Blood +	+	+++
Vera Cruz (Pérez Grovas)					
8. Case 2 (recovered)	2 days and a fraction		Blood +	+	+
Morropón (Noguchi and Kligler)					
9. Case 10 (recovered)	1 day and a fraction		Not practicable	+	
10. " 11	2 days and a fraction		" "	+	
11. " 12 (died)	2 " " "		" "	+	
12. " 13 (recovered)	2 " "		" "		
Vera Cruz (Le Blanc)					
13. Case 2 (recovered)	1 day and a fraction		Blood + (confirmed on third passage)		++
Palmeiras (Vianna and Bião)					
14. Case 3 (recovered)	Less than 1 day		Not practicable	+	
15. " 5	1 day and a fraction		" "	+	

The ætiological relation of *L. icteroides* to yellow fever can be shown also by Pfeiffer tests, with cultures of the organism, of the serum of cases already in the convalescent period. Pérez Grovas (10) found the Pfeiffer reaction with *L. icteroides* constantly present in seventeen convalescents from yellow fever. The American-Brazilian Yellow Fever Commission (7) made Pfeiffer tests of the serum of fourteen cases of yellow fever in Brazil against *L. icteroides* from sources in Ecuador, Mexico, Peru, and Brazil, and obtained positive reactions in all. Parallel reactions with *L. icterohæmorrhagiæ* were negative, as were also Pfeiffer tests of normal sera. Pothier and Pareja (11), in Colombia, in 1923, obtained positive Pfeiffer reactions with *L. icteroides* and negative with *L. icterohæmorrhagiæ* in four persons recovering from a disease suspected of having been yellow fever, the clinical diagnosis being thus confirmed.[2]

Adaptation of Leptospira icteroides to Experimental Animals.

Young guinea-pigs, which are the only animals so far found to be useful in the transmission of yellow fever directly from human cases, are, however, rather refractory at best, while fully grown guinea-pigs are so resistant that even adapted guinea-pig passage strains fail to infect them. Even in young guinea-pigs a clear-cut, typical infection, with hæmorrhages, jaundice, and nephritis after three to six days incubation, is not always induced by inoculation of blood from yellow fever patients, but a suggestive febrile reaction and capillary injection of the conjunctivæ, after a relatively long interval,[3] may be the only symptoms noticed. The period of incubation in the first generation in guinea-pigs has occasionally been as long as eight, nine, or even ten days, and only a few animals among many receiving the same blood may react to the inoculation. If the mild infection at first induced is allowed to run its course, the animals completely recover, but if the blood, or a suspension of liver and kidney, taken at the height of fever,

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[2] Recently Muller and Blaisdell have obtained positive Pfeiffer reactions with the sera of persons who had yellow fever during the Salvador epidemic of 1924.

[3] It may be mentioned here that in Rocky Mountain spotted fever, the incubation period in guinea-pigs is between three and six days, but in the case of a weakened guinea-pig passage virus it may be as long as sixteen days. Such a virus regains its usual virulence on subsequent transfers to guinea-pigs.

is inoculated into several young guinea-pigs, the febrile reaction may occur sooner and the other symptoms of yellow fever become more distinct in some of the animals. A fatal infection is sometimes induced in the second generation, but it may be necessary to make further passages before the strain acquires sufficient virulence for the guinea-pig to induce typical fatal infection. Autopsy shows the characteristic petechial spots in the lungs, hæmorrhages into the gastro-intestinal tract, acute nephritis, and yellowish-brown liver.

In the early experiments in Guayaquil, the necessity of timely transfer from mildly infected to new animals was not realized, and the opportunity of obtaining a larger number of strains of the organisms was thus lost. Of the twenty-seven cases studied, there were five which furnished strains of moderate initial virulence, one which required three passages in the guinea-pig to induce a fatal infection, and nine others which induced an initial disease so mild that the opportunity for transfer was lost before the nature of the infection was realized. That these animals (sixteen in all), which showed only a slight febrile reaction and a trace of jaundice, actually passed through a mild infection with *L. icteroides* was subsequently proven by their immunity to massive doses of adapted strains of the organism or by the characteristic hæmorrhagic spots in the lungs revealed by examination after recovery. Injection of blood from patients suffering from diseases other than yellow fever does not confer immunity against *L. icteroides*, nor produce the petechial lung hæmorrhages, in guinea-pigs.

L. icteroides is essentially a human pathogen, but when fully adapted to the guinea-pig its virulence for this animal may reach such a titre that 0.00001 c.c. of a suspension of liver or kidney of an infected animal, or of culture, will induce a fatal infection, with characteristic hæmorrhages, jaundice, and nephritis. The incubation period becomes fixed to three to six days, as in human yellow fever.[4]

Guinea-pig passage strains of *L. icteroides* are virulent also for young puppies, marmosets, and "prego" monkeys (*Cebus macrocephalus*),

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[4] That the incubation period in human yellow fever may be longer than six days was observed by the French Commission to Brazil (*Ann. de l'Inst. Pasteur*, 1903, xvii, 665), who described two instances of experimental, and three of natural infection, in which the incubation period was estimated at twelve to thirteen days.

and when used in massive quantities, representing several thousand minimum lethal guinea-pig doses, induce symptoms and lesions more strikingly like those of human yellow fever: intense jaundice, anuria, albuminuria, and "black vomit." Guinea-pigs also invariably show effused dark-coloured blood in the stomach, and hæmorrhages into the intestines are common. Autopsy reveals fatty liver, congested kidneys, and normal spleen. The histopathological changes in experimental yellow fever have been studied in detail by Perrin (12) and Muller (13). The reader is referred also to the recent work of W. H. Hoffmann in Havana (14). All these investigators have concluded that the pathological changes induced in experimental animals by *L. icteroides* are identical with those of human yellow fever.

Muller (13) has studied also the hæmatological findings in experimental yellow fever and confirmed the earlier observations (15). There is moderate leucopænia, comparatively slight reduction in hæmoglobin and red blood cells or none at all, as in human yellow fever (2), (16), (30).

One of the proverbial symptoms of yellow fever is the so-called Faget's sign, or relatively slow pulse with high temperature. Cohn and I (17) studied the effect of *L. icteroides* infection on guinea-pigs and marmosets by means of electrocardiographic records and found that the same phenomenon appeared in experimental animals. Relative brachycardia is observed also in human infectious jaundice, and in experimental infection with *L. icterohæmorrhagiæ.*

The Filterability of Leptospira icteroides.

Reed and Carroll (18), Rosenau, Parker, Francis, and Beyer (19), Marchoux, Salimbeni, and Simond (20), showed that the virus of yellow fever passes through the pores of Berkefeld filters. In the early experiments in Guayaquil (21), it was found possible to infect guinea-pigs with the filtrate obtained by passing dilute infective liver and kidney suspension through Berkefeld filters, and subsequent experiments (Nichols (22), Dieterich (23), the American-Brazilian Yellow Fever Commission (7)) have shown that *L. icteroides*, whether in infected organ suspensions or in culture, is capable of passing through Berkefeld filters of the V and N grades. It is of special interest that Nichols used in his filtration experiments the original

filters employed by Walter Reed and his associates in their historic experiments in Havana.

The filterability of *L. icteroides* conforms with one of the most important characteristics of the yellow fever virus, and in this respect *L. icteroides* differs from all organisms previously thought to be the cause of yellow fever.

Cultural Properties of Leptospira icteroides.

An interesting point in connection with the cultivation of *L. icteroides* is the adjustment of reaction of the culture medium. In an earlier publication (21) it was stated that growth of the organisms occurs in the "leptospira medium"[5] when the hydrogen-ion concentration is between pH 6 and pH 7.4. The question has recently been restudied with the collaboration of Dr. Telémaco Battistini (24), not only with *L. icteroides*, but also with *L. icterohæmorrhagiæ* and *L. hebdomadis*, and it has been found that all three species of leptospiras grow well between pH 5.45 and pH 7.21, but not at all at pH 4.65 or pH 8.19. The *icteroides* undergoes degenerative changes much sooner at pH 7.21 than at 5.45 to 6.26, while *L. icterohæmorrhagiæ* and *L. hebdomadis* are as active at pH 7.21 as at pH 5.45. It is evident, therefore, that *L. icteroides* prefers slight acidity and scarcely tolerates an alkalinity beyond pH 7.21. In view of the alkalinity which may be introduced into carefully adjusted media by the use of ordinary soft glass test-tubes, particularly when they have been used for some time, it is well to adjust the culture medium to a point near pH 6 instead of pH 7.4, as formerly recommended. Many of the irregular results previously obtained in the cultivation of *L. icteroides* are undoubtedly explained on the basis of unsuitable reaction of the culture medium employed.

In the light of the exact determination of the hydrogen-ion concentration for the growth of *L. icteroides*, and of the recent work of

[5] The formula for this medium is as follows:

0.9 per cent. NaCl	800 parts
Fresh rabbit serum	100 "
2 per cent. nutrient agar (pH 6.5–7.0)	100 "
Rabbit hæmoglobin (made by laking one part of the defibrinated blood with three parts of distilled water)	10–20 "

Rous (25) on the pH of various animal tissues, it becomes evident why the organism does not multiply in the blood or body fluids of which the pH ranges from 7.6 to 8.2, as in the case of man and some animals. Rous found that the pH of the liver and the kidney in a mammalian (mouse), on the other hand, varies from 5.4 to 6.6, hence they offer an optimum reaction for the multiplication of *L. icteroides*. The particular attack of yellow fever on these two organs may perhaps be partly explained by this factor. *L. icterohæmorrhagiæ* shows similar, though less pronounced, predilections. All the organs or tissues spared by yellow fever have a pH either too acid or too alkaline for the survival and multiplication of *L. icteroides*. The pH range of *L. icteroides*, in comparison with that of most pathogenic bacteria, is rather narrow.

The disappearance of *L. icteroides* from the liver and kidney of experimental animals (young guinea-pigs, puppies, and certain monkeys) is proportional to the degree of histological (hence functional) changes induced by the invading organisms. The more profound the changes, the fewer the surviving organisms, until none at all can be found when the injuries reach a certain limit. The cessation of physiological function of the involved organs and consequent lack of nutrient substance lead to the total disappearance of the *icteroides* towards the later stage of a fatal infection. The phenomenon is perhaps somewhat more constant and rapid in human yellow fever than in experimental animals, but it is marked in the monkey, *C. macrocephalus*, in which *L. icteroides* induces an infection comparable in every respect to human yellow fever (7), (13). The disappearance of *L. icteroides* from the entire system in the case of a non-fatal infection is obviously due to the appearance of specific immune substances resulting from the infection.

Differentiation of Leptospira icteroides from L. icterohæmorrhagiæ and L. hebdomadis.

While a few clinicians (26), (27) regard that yellow fever and infectious jaundice are quite dissimilar in clinical manifestations, the majority of those who have had experience with both diseases agree that the essential features are similar (2), (28), (29). Both are characterized by sudden onset, capillary suffusion of the face and con-

junctivæ at the beginning of fever, severe headache, backache, and muscular pains in the limbs, nausea, early epistaxis, etc., the preliminary symptoms leading in both diseases to jaundice, acute nephritis, and hæmorrhages. The so-called Faget's sign, or relatively slow pulse with high temperature, is present in both diseases. Herpes labialis,[6] considered by some as almost pathognomonic for infectious jaundice, is also observed in yellow fever patients (21).

Some have laid stress upon the paler character of the jaundice in yellow fever, but all grades and all shades of colour, from pale lemon to deep orange, have been met with in both diseases. The clinical difference between yellow fever and infectious jaundice appears to be the somewhat slower course and usually less fatal character of the latter infection. In yellow fever death may occur in five to seven days, while in infectious jaundice the greatest mortality is between the ninth and eleventh days. In some cases of infectious jaundice there may be a second rise of temperature towards the end of the second week, but many patients recover without relapse (31). Relapses have also been known to occur in yellow fever, and are regarded as of unfavourable prognosis (32).

Seven-day fever (33) is also characterized by jaundice, hæmorrhages, and nephritis, but all of a far milder degree than in yellow fever or infectious jaundice.

Morphological Differentiation.

The most reliable means of morphological differentiation is the study of cultures under the dark-field microscope. It is understood that for the purpose of comparison the culture media must be identical in composition and hydrogen-ion concentration, and the cultures must be of the same age and grown under the same conditions of temperature. With the cooperation of Dr. Battistini (24) comparative studies of this kind have been made.

L. icteroides is decidedly shorter (4 to 14 μ) and somewhat thinner (0.2 μ) than *L. icterohæmorrhagiæ*. The elementary spirals are less closely set. The shorter forms assume a semioval circle, the longer

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[6] Herpes labialis has now been shown to be an independent infection caused by a filterable virus. It may occur in association with a number of diseases, hence it has no significance for diagnosis.

specimens show greater flexibility, doubling up at any angle. All rotate swiftly, with some lateral vibratory movements.

L. *icterohæmorrhagiæ* is considerably longer than L. *icteroides* (8 to 24 μ), usually more uniform in length, and heavier in aspect. The elementary spirals are more closely set, although in a very long specimen the spirals are somewhat looser. They have the well-known hooked ends and straight bodies, and rotate swiftly and gracefully without lateral vibration.

L. *hebdomadis* averages slightly longer (10 to 30 μ) than L. *icterohæmorrhagiæ*, and the elementary spirals are almost geometrically equidistant and distinct. The organisms rotate swiftly and evenly without relaxation of the elementary spirals and without bending the ends into a hook, the body being almost a straight line in living forms. Killed specimens have the characteristic hooked ends of a leptospira. Occasionally one observes living organisms showing a small hook at one end, but they soon stretch out with change of movement. There is no difficulty in distinguishing this species from the other two.

Stained preparations may also be used for differential purposes. Leptospiras do not stain readily, especially in culture on the semisolid leptospira medium which gives the richest growth. The best results are obtained by fixing the culture organisms in buffered formalin (34) and staining the dried, thin films with a saturated alcoholic solution of basic fuchsin or gentian violet for a few seconds, or with Giemsa's solution (1 in 20 in distilled water) for two hours. Osmic acid fixation of the wet film, followed by staining with Giemsa solution, also gives good results. In well-stained preparations the elementary spirals can be easily recognized. The thin, delicate, rather short hooked forms of L. *icteroides* can be distinguished from the somewhat heavier and longer L. *icterohæmorrhagiæ* with terminal hooks and the rather straight and coarser L. *hebdomadis*. It is essential for the purpose of comparison to use cultures grown under identical conditions and stained in the same dye solution.

I have had opportunity recently to show cultures of the three leptospiras to Professors Inada, Brumpt, Calkins, Iturbe, and others, all of whom recognized the morphological differences between the species.

Immunological Differentiation.

In the earlier experiments (35) it was shown that *L. icteroides* and *L. icterohæmorrhagiæ* can be distinguished by means of monovalent immune sera. Recently Dr. Battistini (24) has undertaken anew a determination of the immunological relations between *L. icteroides*, *L. icterohæmorrhagiæ* and *L. hebdomadis*, growing the organisms on culture media containing 10 and 1 per cent. of monovalent immune rabbit sera. It has been found that the different strains of *L. icteroides* (from Guayaquil, Mexico, Peru, and Brazil) undergo agglutination and disintegration when inoculated into media containing anti-*icteroides* immune sera, but grow well on media containing anti-*icterohæmorrhagiæ* or anti-*hebdomadis* immune sera. Conversely, the strains of *L. icterohæmorrhagiæ* (obtained from sources in the United States (36), Ecuador (37), and Sumatra (38)),[7] undergo similar changes in media containing anti-*icterohæmorrhagiæ* sera, but grow luxuriantly in media containing anti-*icteroides* or anti-*hebdomadis* immune sera. In the same way, anti-*hebdomadis* sera affect only *L. hebdomadis*.[8]

The experimental infection with *L. icteroides*, like human yellow fever, confers a lasting immunity. As has already been mentioned, the serum of persons recently recovered from yellow fever contains certain immune principles which produce the Pfeiffer phenomenon with *L. icteroides*. Parallel reactions with *L. icterohæmorrhagiæ* have been uniformly negative, and normal sera invariably give negative reactions with both organisms. The duration of the Pfeiffer phenomenon has not been determined but is variable in different individuals. Positive reactions were obtained with the sera of five convalescents in Peru (5), and in the case of two persons who had had yellow fever as long previously as ten and eleven months, while in two other persons who had had the disease ten months previously the reaction was incomplete or doubtful. In Guayaquil (40) fifteen of eighteen convalescents or recovered persons gave a positive reaction; in Colombia and

[7] I am indebted for several strains of *L. icterohæmorrhægiæ* to Professor G. Baermann, of Petoemboekan Hospital, Sumatra.

[8] Two strains of *L. hebdomadis* have been obtained through the kindness of Professor R. Inada, Tokyo Imperial University.

Brazil the reaction was positive in all cases studied. Pérez Grovas (10) found that the reaction was not present as early as the eighth day (one case), but was constantly present after the twelfth day (seventeen cases). There are two instances on record of long duration of the Pfeiffer phenomenon. One is that of Dr. Henry Hanson, a sanitarian (41) who had had severe yellow fever four years previously and had remained in an infected area for two years afterwards; a strong positive reaction was obtained with his serum. Dr. Kligler, who had a sharp attack of yellow fever in Morropón in 1920, and recovered promptly following the administration of anti-*icteroides* serum by Dr. Caballero within forty-eight hours of onset, recently found that his serum still gave a mild positive reaction (four and a half years).

Pérez Grovas (10), employing the strain of *L. icteroides* isolated by him in Vera Cruz (4), studied 183 cases of yellow fever for the purpose of determining whether specific agglutinins for *L. icteroides* could be demonstrated, and found that agglutination tests were more sensitive than Pfeiffer reactions, and that the agglutinating properties of the serum in yellow fever are constantly demonstrable, and as early as the beginning of the second week, that is, earlier than the Pfeiffer phenomenon. Sera from twenty-three normal individuals, and from seventeen persons suffering from diseases other than yellow fever, and sera from persons who had had yellow fever years previously, gave negative agglutination reactions. Positive reactions were obtained in seven of eleven vaccinated persons who had received the second injection of vaccine eleven to fifteen days previously.

The various immunological experiments described clearly establish the immunological entity of *L. icteroides*.

The Non-Contagiousness of Infection with Leptospira icteroides.

Like most insect-borne infectious diseases, yellow fever is not transmitted from person to person by direct contact. The excreta and vomitus are not infectious, and only in a few instances out of hundreds of autopsies has it been suspected that a physician performing post-mortem examination has acquired the infection by this means. Generally speaking, the performance of autopsies on yellow fever cadavers is without danger, owing undoubtedly to the disappearance of the virus before death occurs.

L. icteroides behaves much the same with respect to contact as does the virus of yellow fever. Among hundreds of susceptible young guinea-pigs which have been kept in the same pens or cages with fatally infected animals, not a single instance of contact infection has been observed, notwithstanding the animals shared the same food, and the well lived amidst the excreta of the sick. When a fatal *icteroides* infection has been allowed to run its natural course, and the liver and kidneys reveal profound fatty degeneration and necrosis, blood or organs taken at autopsy are seldom infectious, and *L. icteroides* is recovered from such animals only in a small percentage of instances. In the Brazilian monkey, *C. macrocephalus*, in which typical yellow fever was allowed to run its course, no leptospiras could be demonstrated in the material obtained at autopsy.

Ædes ægypti, Yellow Fever, and Leptospira icteroides.

The theory of Finlay that *Ædes ægypti*, formerly called *Stegomyia calopus*, transmits yellow fever from man to man, was first experimentally demonstrated in Havana in 1900–1901 by the Yellow Fever Board of the United States Army under Major Walter Reed. Nonfatal yellow fever was induced in eighteen non-immune volunteers by subjecting them to the bites of mosquitoes twelve days or longer after the insects had bitten patients who were in the early stages of yellow fever (one to three days after onset). A single experiment was made with a mosquito which had sucked the blood of a patient four days after onset of yellow fever, and a negative result obtained, but inasmuch as some mosquitoes which had ingested blood during the first three days of illness failed to infect under the same conditions, the Board did not conclude that the virus is not present in the blood later than three days (42). The French Commission to Brazil obtained negative results in three instances in which blood taken on the fourth day of illness was inoculated into normal persons (20), hence they considered that the virus probably disappears from the blood circulation about this time.

With regard to the incubation period of the virus of yellow fever in the mosquito, that is, the period between the infecting meal and the time when the mosquito actually becomes infective, the report of the Army Board states that the exact length of the period could not be

determined, inasmuch as no experiments were performed with contaminated mosquitoes between the eighth day after the infecting meal, when the result was negative, and the twelfth day, when the mosquitoes were found to have become capable of conveying infection. Carter (50), before the experimental demonstration of mosquito transmission of yellow fever, had determined, by painstaking study of an epidemic in Orwood, Miss., that there was a definite interval between the occurrence of yellow fever in a given house and the time when the house became a source of infection. When all other sources of infection could be excluded, he found that the interval between the first (primary) case of yellow fever and the secondary cases in a given house varied between eleven and a half and twenty-three days. Hence, when we subtract the period between exposure and onset, about three days at the shortest, the minimum period of incubation of the virus in the mosquito becomes about eight and a half days. Reed (43), in comparing the Board's experimental results with the empiric observations of Carter, expressed the opinion that the minimum period of mosquito incubation might be about ten days. Notwithstanding these definite indications that the period is as short as eight and a half to ten days, however, it is usually referred to in the literature as twelve days.

When *L. icteroides* was first isolated in Guayaquil, the question of mosquito transmission of the organism immediately suggested itself, and experiments were undertaken to determine whether the experimental, as well as the human, infection could be transmitted by *A. ægypti* (44). Six experiments were carried out with mosquitoes infected from human beings, and in one of the six (in which eight insects were used) typical experimental yellow fever was induced in guinea-pigs by the bite of mosquitoes fed twenty-three days previously on a yellow fever patient. In a parallel series of experiments in which the insects were fed on experimentally infected animals, two guinea-pigs developed the typical infection, one of which had been bitten by nineteen mosquitoes infected eight and twelve days previously, and the other by eighty-three mosquitoes fed eight days previously. Twenty-five mosquitoes of the latter lot were emulsified in Ringer solution two days later and the entire suspension, in which a small number of leptospiras was demonstrated by dark-field exam-

ination, inoculated into a young guinea-pig. The animal developed typical infection and died eight days later. *L. icteroides* was demonstrated in all of the animals infected by mosquitoes. Another group of the infected mosquitoes was placed in 10 per cent. formalin and brought back to The Rockefeller Institute, but impregnation of the mosquito tissues with silver nitrate made them so powdery that they were useless, hence the study of the distribution of the leptospira in the mosquito body had to be postponed until opportunity for further study should present itself.

The successful transmission of experimental yellow fever from guinea-pig to guinea-pig by means of mosquitoes which, in one instance, had had their infective feeding only eight days previously, cannot be regarded as conflicting with the data obtained in man, for reasons already stated.

Iglesias (45), head of the Vera Cruz yellow fever laboratories of the Mexican Department of Health, was successful in infecting mosquitoes by feeding them on an infected guinea-pig and in transmitting the infection subsequently from the mosquitoes to normal guinea-pigs. Two animals, exposed to the bites of the infected mosquitoes twenty-three and thirty-one days, respectively, after their infecting meal, showed suggestive symptoms of mild experimental yellow fever, and in the transfer from one of them the leptospira was demonstrated in the blood.

The experiments so far made are sufficient to establish the fact that the virus of yellow fever and *L. icteroides* are alike transmissible by *A. ægypti*.

Application of Immunity Phenomena to Prophylaxis and Serum Treatment of Yellow Fever.

As is well known, there are a few instances in which killed cultures of pathogenic micro-organisms are capable of inducing immunity to subsequent infection with virulent strains. An attack of yellow fever confers lasting immunity, and the question at once arose whether the injection of killed cultures of *L. icteroides* would induce appreciable protection against the living organism.

Experiments on guinea-pigs were undertaken by Pareja and myself (46) immediately after the isolation of *L. icteroides* in Guayaquil, and

it was found that animals which had received killed cultures resisted subsequent infection. In view of this result, we were asked to inoculate a large number of non-immune soldiers who were to be transferred from non-infected regions to Guayaquil. Of 149 soldiers vaccinated in Quito, 29 received 2 c.c. of killed cultures, the remainder 1 c.c. Among the latter group three later contracted yellow fever. Among another group of 176 soldiers and their families, vaccinated after arrival in Guayaquil with a vaccine preparation about one-fifth the strength of that used in Quito, two cases of yellow fever developed. One may be regarded as a control, in that it developed shortly after vaccination, before immunity could have developed.

The relatively small incidence of yellow fever among the vaccinated group, as compared with that among the other non-immune population of Guayaquil, indicated that it was desirable to continue trial of the vaccine, and subsequent preparations have been of a strength such that one cubic centimetre contains 2,000,000,000 leptospiras, as compared with 2,000,000 in the earlier preparation. Two injections of 2 c.c. of the vaccine are administered whenever possible. This material has been used extensively in Mexico, Peru, Salvador, Guatemala, British Honduras, Brazil, and the West Coast of Africa, more than 20,000 individuals having been vaccinated. Although in many instances the vaccinated were not subsequently exposed to yellow fever, owing to the decline of the epidemic or to their having left the epidemic area, there were nevertheless a large number whose immunity was subjected to test. Table II shows the relative case incidence of yellow fever among vaccinated and unvaccinated in various epidemics during the course of the past six years. There are two groups, those who contracted yellow fever before sufficient time had elapsed after vaccination for immunity to develop (a period of ten to fifteen days being required), and those taken ill later than this period. Although many cases have occurred during the pre-immunity period, only rarely has a vaccinated person contracted yellow fever later, notwithstanding the high case-incidence among unvaccinated during the same period. The pre-immunity cases may be regarded as controls, and the occurrence of yellow fever among them as showing that vaccinated persons were exposed to yellow fever equally with non-vaccinated, while the abrupt drop in incidence among vaccinated after the fifteen-day

TABLE II.

Vaccination against Yellow Fever.*

Place and population	Year	Number vaccinated and physician in charge	Number cases yellow fever among vaccinated				Number cases yellow fever among unvaccinated during same period	Incidence	
			Before 10 days†		After 10 days			Among vac.	Among unvac.
			1 inj.	2 inj.	1 inj.	2 inj.			
Ecuador‡									
Quito§	1918	Noguchi and Pareja 149	0	0	3‖	0)	386	11 per 1,000	110 per 1,000
Guayaquil		176	1	0	2¶	0)			
		Board of Health 102	0	0	0	0)			
Honduras									
U.S.S. Chicago	1919	Lyster, Pareja, and Bailey 75	0	0	0	0			
Amapala	1919	425	0	0	0	0			
Salvador 113,000	1920	Lyster and Bailey 3,469 (1 inj.) 138 (2 inj.)	5	0	5**	0	181		
Guatemala	1920	Lyster and Vaughn 791 (1 inj.) 592 (2 inj.)	1	1	0	0			
Mexico		Lynn and Guadarrama							
Tuxpam, 6,000	1920	2,000 (2 inj.)	0	17	0	0	85	0	{21.3 per 1,000
Vera Cruz	1920	Board of Health 514 (2 inj.) 234 (1 inj.)	2	0	3‡‡	0	199		
Manzanillo	1921	Surg.-Gen. Osornio 600	0	0	0	0	"Numerous"		

Locality	Year	Observer	Number vaccinated*						
Honduras (Brit.)									
Belize	1921	Gann	146	0	7	0	17	0	3.4%
Peru									
Tambogrande, 500	1920	Noguchi and Kligler	47	0	1	0		0	0
Paijan	1921	Hanson	200	0	0	0	"Numerous"	0	0
Lambayeque			200	0	0	0	"	0	0
Brasil									
Ceará (D. P. Robinson Co.)	1922–23	Broad St. Hosp., N. Y.	57	0	0	0	"10 per month"	0	0
		Thomas	68	0	3	1††	22		

* These figures include only persons vaccinated while in an endemic or epidemic area, or about to enter such an area, that is, persons who were subject to exposure to yellow fever after vaccination.

† The cases recorded in this column were in persons who were already in the epidemic area when vaccinated, and who contracted yellow fever before there had been sufficient time (ten to fifteen days) for the development of immunity.

‡ The vaccine employed during these preliminary experiments in Ecuador contained about 2,000,000 organisms per c.c., while that used on all subsequent occasions was a thousandfold stronger (2,000,000,000 organisms per c.c.).

§ The vaccinations were carried out in Quito on September 24, 1918, and the vaccinated troops arrived in the epidemic area (Guayaquil) on October 7.

‖ Received 1 c.c. only.

¶ The supply of vaccine being almost exhausted, the remainder was diluted. In this instance only 1 c.c. of the diluted vaccine was injected.

** Incomplete vaccination (1 injection only). In two of these cases the diagnosis was doubtful.

†† This is the only case recorded in which yellow fever occurred notwithstanding two injections of vaccine and a lapse of time ample for development of immunity. The patient received anti-*icteroides* serum on the second and third days of illness and recovered.

‡‡ Incomplete vaccination (1 injection only).

period, notwithstanding continued occurrence of cases among un-
vaccinated, indicates that the vaccination confers protection against
infection.   The protection induced by vaccination lasts about five
to six months.

The presence of immune substances in the serum of persons who
have recovered from yellow fever, as shown by the Pfeiffer reaction,
is not so permanent as the immunity of the body as a whole.   Persons
who have had yellow fever years ago remain immune, though their
serum may not give a positive Pfeiffer reaction, hence the immunity
induced by natural infection must be due to at least two factors, one
humoral, more transient, the other histogenous and lasting.   Only the
former is transferable to non-immune persons, and the serum of yellow
fever convalescents is useful for treatment in proportion to the amount
of immune substance present (20), (39).

It is possible, however, to produce immune substances artificially
in enormous quantity in the serum of certain naturally less susceptible
animals by repeated injections at regular intervals of living cultures of
L. icteroides.   During the past several years we have been able to pre-
pare[9] in horses an immune serum such that a quantity as small as
0.0001 c.c. is capable of protecting a guinea-pig against 1,000 minimum
killing doses of *L. icteroides*, when given simultaneously.   Such po-
tency is never found in the serum of yellow fever convalescents.
Somewhat larger quantities of the serum save guinea-pigs already
infected, when administered during the early period (the first three
days) of illness.   There was sufficient justification, therefore, for em-
ploying this immune serum in the treatment of yellow fever in man.

The first use of the anti-*icteroides* serum in human yellow fever was
by Lyster, Pareja, and Bailey in 1919, and favorable results were
reported (47).   Treatment was continued by Lyster and his associates
in the Central American countries and in Mexico, in British Honduras
by Cran (48), and later in various localities.   The results are sum-
marized in Table III.

The statistics clearly indicate the efficacy of the serum when ad-
ministered during the first three days of illness, the mortality being 16
per cent. as against 50 to 100 per cent. during the same epidemic

[9] This work has been conducted under my direction by Mr. Julius Klosterman.

TABLE III.

Serum Treatment of Yellow Fever.

Place	Year and physician in charge	Serum administered before fourth day			Serum administered after fourth day			Mortality among untreated cases during same period
		Total	Rec.	Died	Total	Rec.	Died	
Salvador	Lyster and Bailey, 1920	14	11	3*	28	15	13	51 per cent.
Guatemala	Lyster and Vaughn, 1920	3	3	0	1	0	1	68 "
Honduras	Lyster, Pareja, and Bailey, 1919	1	1	0	–	–	–	
Honduras (Brit.)	Gann, Cran, 1921	12	11	1	4	0	4	
Mexico								
Mérida	Hernandez, 1920	4	4	0	4	0	4	80 "
Vera Cruz	Board of Health, 1920	16	16	0	3	0	3	49 "
Gutierrez-Zamora	Le Blanc's Report, 1920–1921	17	17	0	1	0	1	3 cases untreated, all died.
Tuxpam	Lynn and Guadarrama, 1920	36	27	9	38	21	17	68.8 per cent.
Peru	Noguchi and Kligler, 1920	4	3	1†	–	–	–	"Up to 100 per cent."
"	Hanson, 1921	8	6	2‡	–	–	–	
Brazil	Barreto, 1920	4	2	2	2	1	1	
"	Borges Vieira, 1920	–	–	–	1	0	1	
"	Cavalcanti, 1920	12	11	1	5	4	1	
"	Thomas, 1923	12	8	4	–	–	–	
West Africa								
Nigeria	Leonard, 1922	1	1	0	–	–	–	1 case untreated, died.
Grand Bassam	Bauvallet, 1922	4	3	1	2	0	2	
Total		148	124	24	89	41	48	

* Received total of 20 c.c. in two days—"too small an amount to have any material effect" (Dr. Bailey).

† Patient subjected to forced journey during first two days of illness; disease extremely severe; patient exhausted, with severe nephritis, when admitted to hospital.

‡ Day of disease on which serum was given not recorded.

period among cases treated after three days or not treated with serum at all.

It is understood, of course, that serum treatment must be accompanied by reasonable symptomatic treatment. It is well known that undue physical exertion may bring about a fatal termination even in mild yellow fever, and that rest, proper nursing, and suitable diet (Carter recommends no food but plenty of water) are essential (49). The effect of the serum is indicated, according to those who have used it, not only by the reduction in mortality but in the prompt alleviation of symptoms (6).

Relation of Vaccination and Serum Treatment to Sanitary Regulations.

When specific prophylaxis and serum treatment for yellow fever were suggested, special emphasis was placed upon the fact that they were supplementary means of fighting the disease and would not supplant the method of elimination by mosquito control (51). They were intended, the one to give non-immune persons temporary protection until the mosquito index is sufficiently reduced for safety, the other to give the patient the benefit of a curative serum without neglect of the usual symptomatic (52).

Protection induced by the use of killed micro-organisms is, in general, not so durable or complete as that brought about by spontaneous infection or by modified live virus such as is used for small-pox vaccination. Moreover, the proof of effective cow-pox vaccination can always be seen in the local manifestations, and the presence or absence of protection against small-pox in a vaccinated person is readily determined by the outcome of subsequent vaccination. Hence a certificate of small-pox vaccination can very well be accepted in lieu of quarantine. In yellow fever[10] we have no parallel procedure to which we may resort in order to prove that all vaccinated individuals are protected. But since susceptible animals become resistant to the *icteroides* infection after inoculation of killed cultures, and since the blood serum of vaccinated persons acquires protective property against *L. icteroides*, as shown by tests in guinea-pigs, it is assumed that vaccinated persons are protected, and this assumption seems

[10] The basis for yellow fever vaccination is analogous to that for vaccination against typhoid fever, and the same general laws apply to both.

to be well borne out by the actual results obtained with yellow fever vaccine in several thousand human beings, as already described. Results analogous to those obtained in yellow fever, and also decidedly favourable, have been reported by Inada on the use of vaccine and serum against infectious jaundice in Japan (54).

The efficacy of vaccination against yellow fever has therefore been substantiated, as was that of antityphoid vaccination, and its use in combination with other prophylactic measures has been generally adopted. It may be said, as was said by Russell (53) in connection with antityphoid vaccination, "there is no occasion for conflict between the advocates of general and individual prophylaxis; one is as necessary as the other, and no one interested in the suppression of this disease can afford to ignore either."

SUMMARY.

The properties of *L. icteroides* are the same as those already demonstrated for the virus of yellow fever: it passes through bacterial filters; it is killed by a temperature of 50°C. maintained for five minutes; it is transmissible from man to animal and from animal to animal by means of infected female *A. ægypti* mosquitoes. *L. icteroides* is invisible under the ordinary microscope but visible by dark-ground illumination.

L. icteroides induces a symptom complex and pathological changes in suitable experimental animals (young guinea-pigs, puppies, and certain monkeys) which are identical with those of human yellow fever.

Yellow fever and infectious jaundice are distinct, independent infections. They are very similar in character.

L. icteroides is distinct, both morphologically and serologically, from *L. icterohæmorrhagiæ* of infectious jaundice and *L. hebdomadis* of seven-day fever.

The prophylactic value of the vaccine prepared with killed cultures of *L. icteroides* and the efficacy of the anti-*icteroides* immune serum prepared in horses were demonstrated in animal experiments and have been substantiated by the results of vaccination and serum treatment in human beings.

The evidences just summarized appear to warrant the conclusion that *L. icteroides* is the cause of yellow fever.

There is no reason for modification of any of the established measures of general prophylaxis or sanitary regulations now in force against yellow fever; specific prophylactic measures and serum treatment should be employed simultaneously with the anti-mosquito campaign and the proper symptomatic treatment of yellow fever.

REFERENCES.

(1) NOGUCHI, H., *Journ. Exper. Med.*, 1919, vol. xxix, pp. 565, 585; vol. xxx, pp. 1, 9, 13, 87, 95, 401; vol. xxxi, pp. 135, 159; vol. xxxii, p. 381.

(2) ELLIOTT, C. A., *Arch. Inter. Med.*, 1920, vol. xxv, p. 174.

(3) NOGUCHI, H., and KLIGLER, I. J., *Journ. Exper. Med.*, 1920, vol. xxxii, pp. 601, 627.

(4) PÉREZ GROVAS, P., *Journ. Amer. Med. Assoc.*, 1921, vol. lxxvi, p. 362.

(5) NOGUCHI, H., and KLIGLER, I. J., *Journ. Exper. Med.*, 1921, vol. xxxiii, pp. 239, 253.

(6) LE BLANC, T. J., in press.

(7) NOGUCHI, H., MULLER, H. R., TORRES, O., SILVA, F., MARTINS, H., RIBEIRO DOS SANTOS, A., VIANNA, G., and BIÃO, M., Monograph 20, Rockefeller Institute for Medical Research, New York, 1924.

(8) LEBREDO, M. G., *Bol. d. Sanidad y Beneficencia*, quoted by Agramonte, A., JOURN. TROP. MED. AND HYG., 1924, vol. xxvii, p. 285.

(9) BORGES VIEIRA, F., *Bol. Soc. Med. e Cir. S. Paulo*, 1921, vol. iv, 2nd ser., p. 137.

(10) PÉREZ GROVAS, P., *Amer. Journ. Trop. Med.*, 1923, vol. iij, p. 325.

(11) POTHIER, O. L., and PAREJA, W., unpublished observations.

(12) PERRIN, T. G., *Amer. Journ. Trop. Med.*, 1923, vol. iii, p. 27.

(13) MULLER, H. R., *Journ. Trop. Med. and Hyg.*, 1924, vol. xxvii, p. 299.

(14) HOFFMANN, W. H., *Journ. Trop. Med. and Hyg.*, 1924, vol. xxvii, p. 235.

(15) NOGUCHI, H., *Journ. Exper. Med.*, 1919, vol. xxix, p. 585.

(16) FROES, J. A., "Hemodiagnostico nos Tropicos," Bittencourt, E.P.M., These: "Hematologia da febre amarella," Bahia, 1915.

(17) COHN, A. E., and NOGUCHI, H., *Journ. Exper. Med.*, 1921, vol. xxxiii, p. 683.

(18) REED, W., and CARROLL, J., *Senate Doc.* No. 822, 61st Cong., 3rd sess., p. 149.

(19) ROSENAU, M. J., PARKER, H. B., FRANCIS, E., and BEYER, G. E., *Yellow Fever Inst. Bull.*, 1905, No. 14.

(20) MARCHOUX, E., SALIMBENI, A., and SIMOND, P. L., *Ann. Inst. Pasteur*, 1903, vol. xvii, p. 665; 1906, vol. xx, pp. 16, 104, 161.

(21) NOGUCHI, H., *Journ. Exper. Med.*, 1919, vol. xxx, p. 13.

(22) NICHOLS, H. J., "Proc. Internat. Conference on Health Problems in Tropical America," Kingston, Jamaica, July 22 to August 7, 1924, p. 221.

(23) DIETERICH, F. H., *Amer. Journ. Trop. Med.*, 1924, vol. iv, p. 553.

(24) To be published shortly.

(25) Rous, P., *Science*, 1924, vol. lx, p. 363.
(26) Guiteras, J., *Sanidad y Beneficencia*, 1921, vol. xxv, p. 34; *Arch. of Diag.*, 1921–22, vol. xiv, p. 1.
(27) Agramonte, A., *Journ. Trop. Med. and Hyg.*, 1924, vol. xxvii, p. 285.
(28) Ryle, J. A., *Quart. Journ. Med.*, 1921, vol. xiv, p. 139.
(29) Carter, H. R., "Proc. Internat. Conference on Health Problems in Tropical America," Kingston, Jamaica, July 22 to August 7, 1924, p. 211.
(30) Carter, H. R., Archibald and Byam's "Practice of Medicine in the Tropics," vol. ii, p. 1228.
(31) Stokes, A., Ryle, J. A., and Tytler, W. H., *Lancet*, 1917, January 27, p. 142.
(32) Manson, P., "Tropical Diseases," W. Wood and Co., New York, 1918, p. 263.
(33) Ido, Y., Ito, H., and Wani, H., *Journ. Exper. Med.*, 1918, vol. xxviii, p. 435.
(34) Noguchi, H., *Journ. Amer. Med., Assoc.*, 1921, vol. lxxvii, p. 2052.
(35) Noguchi, H., *Journ. Exper. Med.*, 1920, vol. xxxi, p. 135.
(36) Noguchi, H., *Journ. Exper. Med.*, 1917, vol. xxv, p. 755.
(37) Noguchi, H., *Journ. Exper. Med.*, 1919, vol. xxx, p. 95.
(38) Baermann, G., *Geneesk. tijdschr. v. Nederlandsch. Ind.*, 1923, vol. lxiii, p. 885.
(39) Agramonte, A., quoted by Finlay, C., "Trabajos Selectos," Havana, 1912, pp. 512–515.
(40) Noguchi, H., *Journ. Exper. Med.*, 1919, vol. xxx, p. 9.
(41) Noguchi, H., *Amer. Journ. Trop. Med.*, 1924, vol. iv, p. 131.
(42) Reed, W., Carroll, J., and Agramonte, A., *Senate Doc.*, No. 822, 61st Cong., 3rd sess., p. 118.
(43) Reed, W., *Senate Doc.* No. 822, 61st Cong., 3rd sess., p. 107.
(44) Noguchi, H., *Journ. Exper. Med.*, 1919, vol. xxx, p. 401.
(45) Personal communication.
(46) Noguchi, H., and Pareja, W., *Journ. Amer. Med. Assoc.*, 1921, vol. lxxvi, p. 96.
(47) Wilson, T., *U. S. Nav. Med. Bull.*, 1920, vol. xiv, p. 200.
(48) Cran, J., "Proc. Internat. Conference on Health Problems in Tropical America," Kingston, Jamaica, July 22 to August 7, 1924, p. 194; Journ. Trop. Med. and Hyg., 1925, vol. xxviii, p. 26.
(49) Carter, H. R., "Nelson's Loose Leaf Medicine, " vol. ii, p. 113.
(50) Carter, H. R., *New Orleans Med. Journ.*, 1900, vol. lii, p. 617; *Med. Record*, 1901, vol. lix, p. 933.
(51) Noguchi, H., *Journ. Amer. Med. Assoc.*, 1921, vol. lxxvii, p. 181.
(52) Noguchi, H., *Lancet*, 1922, vol. ccii, p. 1185.
(53) Russell, F. F., *Amer. Journ. Med. Sciences*, 1913, vol. cxlvi, p. 803.
(54) Inada, R., *Japan Med. World*, 1922, vol. ii, p. 189.

THE IMMUNOLOGICAL RELATIONSHIPS OF THE LEPTOSPIRA GROUP OF SPIROCHÆTES.[1]

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Noguchi, in his study of the immunological relation between *Leptospira icteroides*, of yellow fever, and *L. ictero-hæmorrhagiæ*, of infectious jaundice (1), carried out agglutination, complement-fixation, and Pfeiffer reactions with the sera of experimentally-infected animals and demonstrated that these organisms are serologically distinct. Tests of the protective properties of monovalent, as well as polyvalent, anti-*icteroides* and anti-*ictero-hæmorrhagiæ* immune sera always showed the immunological identity of each of the two species. The results of parallel Pfeiffer tests of the sera of convalescents from yellow fever against *L. icteroides* and *L. ictero-hæmorrhagiæ* have shown a specific immunological reaction of such sera to *L. icteroides* but none to *L. ictero-hæmorrhagiæ* (2). Pérez Grovas (3), in Vera Cruz, and others in later epidemics (2, 4, 5) confirmed Noguchi's results.

Noguchi has recently studied the serological relationship between the different species of leishmania (6) and found that the type of growth obtained on culture media into which monovalent immune sera had been incorporated furnished a simple and precise method of differentiation of species. This procedure, which is similar to that used by Oba (7) in testing the effect of immune sera *in vitro* upon *L. ictero-hæmorrhagiæ*, has been applied to the study of the three types of *Leptospira* now recognized, *L. icteroides*, *L. ictero-hæmorrhagiæ*, and *L. hebdomadis*[2] (seven-day fever), with the results here reported.

[1] Work done under the auspices of the International Health Board of the Rockefeller Foundation.

[2] The culture of *L. hebdomadis* Strain B was obtained through the kindness of Professor Inada.

Culture	Anti-icteroides sera						Anti-ictero-haemorrhagiae serum		Anti-hebdomadis serum		Control with normal rabbit serum
	Le Blanc		Vera Cruz		Braz. Str. 3		Am. Str. 2		Hebd. Str. B		
	1-20	1-100	1-20	1-100	1-20	1-100	1-20	1-100	1-20	1-100	
Strain of *L. icteroides*—											
Guayaquil Str. 1	−	−	−	−	−	−	+	+	+	+	+
Le Blanc	−	−	−	−	−	−	+	+	+	+	+
Peruvian Str. 2	−	−	−	−	−	−	+	+	+	+	+
Vera Cruz Str.	−	−	−	−	−	−	+	+	+	+	+
Brazilian Str. 3	−	−	−	−	−	−	+	+	+	+	+
Strain of *L. ictero-haemorrhagiae*—											
American Str. 2	+	+	+	+	+	+	−	−	+	+	+
Guayaquil Str. No. 30	+	+	+	+	+	+	−	−	+	+	+
L. C. Str.	+	+	+	+	+	+	−	−	+	+	+
Strain of *L. hebdomadis*—											
Hebdomadis Str. B	+	+	+	+	+	+	+	+	−	−	+

+ = Active growth, as usual.

− = Marked agglutination and degeneration followed by death of the organisms within 120 hours.

Technique.

Monovalent sera specific for *L. icteroides,* *L. ictero-hæmorrhagiæ,* and *L. hebdomadis* were prepared in rabbits by intravenous injections at intervals of five to six days; of gradually increasing doses (0.5, 0.1, 1.5, 2.0, and 2.0 c.c.) of actively growing cultures two to three weeks old. The animals were bled by heart puncture eight days after the last injection. The sera were kept in the ice-box until the time of use, no preservatives being used.

The sera were mixed with the culture medium[3] in concentrations of 1 : 20 and 1 : 100, and the sera-containing media were distributed into small test-tubes in quantities of 2 c.c. The two dilutions of a given monovalent serum were tested against each of the three types of *Leptospira,* a capillary drop of a young, actively growing culture being inoculated in each instance. The tubes were incubated at 26°C., and examinations were made by dark-field microscope at intervals of 48, 72, 96, and 120 hours after inoculation.

Table.

As the table shows, the three monovalent anti-*icteroides* sera tested against five strains of *L. icteroides* produced on the first day marked agglutination and degeneration, followed within four to five days by complete fragmentation of the organisms. On the other hand, these sera had no effect upon the three strains of *L. ictero-hæmorrhagiæ* nor upon the strains of *L. hebdomadis,* all of which grew luxuriantly in the media containing the anti-*icteroides* sera. Conversely, the anti-*ictero-hæmorrhagiæ* serum, while producing agglutination, degeneration, and final destruction of all the strains of *L. ictero-hæmorrhagiæ* within three to four days, had no influence upon the growth of *L. icteroides* or *L. hebdomadis.* The anti-*hebdomadis* serum had a similar agglutinating and degenerating effect upon the *hebdomadis* strain, but none at all upon the strains of *L. icteroides* and *L. ictero-hæmorrhagiæ.*

[3] The formula for Noguchi's *Leptospira* medium is as follows:

NaCl, 0.9 per cent.................................	800 parts
2 per cent, nutrient agar, pH 7.4....................	100 "
Rabbit serum.......................................	100 "
Rabbit hæmoglobin.................................	10–20 "

The hæmoglobin solution is prepared by taking 1 part of rabbit blood with 3 parts of distilled water.

SUMMARY.

The immunological properties of the three known species of *Leptospira* have been studied by cultivating the organisms on media containing monovalent homologous and heterologous immune sera. This method of studying the effect of immune sera permits continuous observation over as long a period as desired and thus diminishes the possibility of misinterpretation resulting from gross observations or a single microscopic examination. It also reduces the chance of possible error due to changes which may occur on standing in mixtures of culture with serum alone.

The results obtained constitute additional evidence of the serological distinctions existing among the three species of *Leptospira*, *L. icteroides*, *L. ictero-hæmorrhagiæ*, and *L. hebdomadis*.

REFERENCES.

(1) NOGUCHI, H. *Journ. Exper. Med.*, 1920, xxxi, 135.
(2) NOGUCHI, H., MULLER, H. R., TORRES, O., SILVA, F., MARTINS, H., RIBEIRO DOS SANTOS, A., VIANNA, G., and BIÃO, M. Monograph 20, Rockefeller Institute for Medical Research, New York, 1924.
(3) PÉREZ GROVAS, P. *Amer. Journ. Trop. Med.*, 1923, iii, 325.
(4) NOGUCHI, H., and KLIGLER, I. J. *Journ. Exper. Med.*, 1920, xxxii, 627.
(5) *Idem. Journ. Exper. Med.*, 1921, xxxiii, 253.
(6) NOGUCHI, H. *Proc. Internat. Conf. on Health Prob. in Trop. Amer.*, Kingston, Jamaica, B. W. I., 1924, July 22 to August 1, 455.
(7) OBA. *Eiseigaku Densenbyogaku Zasshi*, 1921.

THE EFFECT OF OPERATIVE INTERFERENCE WITH THE CERVICAL SYMPATHETIC NERVOUS SYSTEM UPON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

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Previous papers dealing with a transplantable neoplasm of the rabbit have emphasized the conception that variations in the character of the disease are largely due to variations in the mechanisms of reaction and resistance of the host and, furthermore, that this mechanism is an expression of animal metabolism or economy. It has been found, for instance, that the clinical course of the malignant disease may be correlated with the state of the glands of internal secretion as measured by the mass relationships of these organs (1). From our observations upon normal rabbits (2) as well as those infected with *Treponema pallidum* (3) it would appear that the alterations in mass relationship in the endocrine glands of tumor animals possess a functional significance and, furthermore, that as one of the factors in the constitutional organization of the animal, this system of organs occupies an important position in the mechanism of animal resistance. This view was submitted to direct investigation by means of various operative procedures with these organs and it was found that the growth and malignancy of the tumor could be affected in certain instances, notably by complete extirpation of the thyroid, a procedure markedly increasing the severity of the disease (4). It was felt, however, that the relative value of individual endocrine organs was of less importance than the integrity and balance of the system as a whole.

The possibility of a direct or indirect participation by the sympathetic nervous system in the reaction and resistance to the tumor has been considered with regard to the growth of the primary tumor and the subsequent course of the disease. In order to investigate it, various

parts of the sympathetic nervous system were removed before inocula-
tion with the tumor. The cervical portion of the sympathetic system
was selected because of its probable connection with the thyroid as
well as its surgical accessibility.

Material and Methods.

Description of the Cervical Sympathetic Nervous System.—The cervical sympathe-
tic nerve system in the rabbit comprises the upper two ganglia of the sympathetic
chain on either side, together with the connecting trunk and rami of distribution.
The superior ganglion lies at the base of the skull just medial to the internal
carotid artery and vagus; the inferior ganglion is situated at the base of the neck
directly above the origin of the subclavian artery; the trunk follows the course
of the vagus through the neck. By analogy with human anatomy it is assumed
that each ganglion is connected to the central nervous system by centripetal fibers
(grey rami) and by centrifugal fibers (white rami). The grey rami join the
corresponding cord segments in the case of each ganglion but the white rami for
both ganglia enter the sympathetic trunk from the upper thoracic segments.
Each ganglion, also, distributes fibers peripherally to blood vessels, glands, etc.
It is not certain which of them, in the rabbit, is chiefly concerned in supplying the
thyroid gland.

Operative Procedures.—Four types of operations were performed: (1) extirpa-
tion of both superior ganglia ("superior sympathectomy"), (2) extirpation of both
inferior ganglia ("inferior sympathectomy"), (3) extirpation of both superior and
both inferior ganglia and of both cervical sympathetic nerve trunks ("complete
sympathectomy"), (4) division of the sympathetic trunk ("sympathotomy").

Ether anesthesia was used for each operation. No especial preparation was
employed except the omission of the morning feeding on the day of operation.
The hair over the ventral cervical region was clipped and shaved and the skin
washed with 50 per cent alcohol. A midline incision was made, and by careful
dissection in a bloodless field, the sympathetic trunk was isolated at a point just
below the thyroid with retraction of the sternomastoid muscle medially. The
trunk was then followed upwards or downwards to the ganglion to be removed.

Extirpation of the superior ganglion was easily accomplished because of ready
approach and clear identification. It was avulsed, together with such of its fibers
of distribution as came with it. Removal of the inferior ganglion, on the other
hand, was much more difficult, principally because of its close relation to the sub-
clavian artery and to a fine blood vessel plexus, and the approach to it was difficult
also. Manipulation of the ganglion during a dissection was found to set up
cardiodepressor reflexes occasionally resulting in sudden death. The operation
of sympathotomy consisted in division of the sympathetic trunk in a midposition
opposite the lower pole of the thyroid, by excising a small section between ligatures.

Our entire group of complete cervical sympathectomy operations numbers

thirty-two rabbits with an operative mortality of 18.8 per cent; however, among the last twenty animals there was but one death.   Twenty-four rabbits have been operated upon for double inferior sympathectomy with an operative mortality of 16.7 per cent and but one death in the last fifteen animals.   There were no deaths in the observation period between these operations and inoculation with the tumor, and we have had no fatalities with the superior cervical sympathectomy or sympathotomy operations.

The criteria of complete removal of the superior or inferior ganglia, or of the sympathetic nerve, were entirely objective, since the conditions of the work precluded the use of physiological tests.   The dissections were so carried out that we could be reasonably certain during the operation of removing the entire ganglion, and its absence was verified by careful search at the postmortem examination of each rabbit.   Extirpation of the cervical nerve or a portion of it was controlled by following the nerve to the superior cervical ganglion before removal or section of it.

Animals.—The animals employed were young adult or adult male rabbits selected and matched according to breed, age, and length of caging.   They were separately caged and fed the ordinary diet of oats, hay, and cabbage used in these laboratories.

Tumor.—The tumor has been described in detail in previous papers (5, 6). It belongs to the class of epithelial tumors, is easily transplanted by intratesticular inoculation, and gives rise to a primary growth and metastatic growths in practically every tissue and organ of the body.   The course of the disease in individual animals or in different series of animals is variable both as regards the actual outcome of the disease and as regards the course of the primary tumor and the incidence and distribution of metastases.   Some rabbits die within 3 to 4 weeks after inoculation with extensive tumor involvement while others which may have shown a similar primary tumor growth ultimately recover and at postmortem examination show few or no foci of tumor.   All gradations between these extremes are to be found.

Method of Inoculation.—At various intervals after operation inoculations were made with a salt solution suspension of an actively growing primary tumor; 0.3 cc. of the tissue emulsion was injected into one testicle.   A suitable number of normal rabbits were inoculated at the same time to serve as controls.

Conduct of Experiments and Method of Analysis of Results.—Each rabbit was examined at frequent intervals with special reference to the following points: the rate, type of growth, and eventual fate of the primary tumor, the appearance of metastases in superficial parts of the body, and the general physical condition of the animal.   Twice a week the animals were weighed and the primary tumor was measured.   Rabbits which developed a paralysis of the legs or a physical deterioration with emaciation and weakness were killed with ether.   The experiment was terminated 2 months after inoculation, at which time all surviving animals were killed.   In each instance a complete postmortem examination was made with

special attention to the distribution, amount, and character of tumor tissue. In addition, all the principal organs were weighed.

The data obtained were used as a basis for estimating the character of the disease. The total probable mortality rate of a group includes those animals which died or were killed during the experimental period of 2 months and those which showed, at the end of this period, such metastatic growths as would probably have caused death at some future time as, for instance, tumors of both suprarenal glands. The incidence and distribution of secondary growths have been considered upon a relative and actual basis, the former including all animals of a group, while the latter takes into account only those in which metastases were found. The percentage estimations of the distribution of metastases have been calculated upon the basis of the number of foci theoretically possible as shown by the actual location of metastases in the first twenty generations of tumor animals (6).

The figures refer to the number of organs or tissues involved, not to the actual numbers of secondary growths, and, consequently, the expressions "foci of metastases," "distribution of metastases," or "metastatic rate" are used rather than "number of metastases." By contrast the figure for the number of clinical metastases detected during the life of the animal refers to the actual number of individual secondary tumors found.

There are certain obvious objections to the above method of estimation. For instance, such organs as the liver and kidneys may be markedly involved with numerous tumors or by only a few which destroy little of the organ. However, the general character of the disease process, whether of high, moderate, or low malignancy, is shown by a grouping of the percentage estimations of metastatic distribution according to the following four subdivisions (Text-figs. 2 and 3): (1) extensions and implantations including those to the retroperitoneal and mediastinal lymph nodes, (2) those to the lungs and pleura, liver, kidneys, and pancreas, (3) to the skin and subcutaneous tissue, superficial lymph nodes, muscles, bones and bone marrow, heart and pericardium, glands of internal secretion with the exception of the suprarenals, spleen, and the central nervous system, (4) to the suprarenals and eyes. This system of grouping was selected for the following reasons.

It has been found, from the study of several hundred rabbits inoculated with this tumor, that in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation there is usually a widespread distribution of metastases to the skin, the superficial lymph nodes, the muscles, the bones and bone marrow, the heart, the spleen, and the endocrine glands as well as to the parenchymatous viscera, the retroperitoneal and mediastinal tissues, and the serous membranes of the abdominal cavity. In instances of a somewhat less malignant disease, the most conspicuous and frequent secondary growths are found in the liver, kidneys, lungs, and pancreas. A level of still lower malignancy is chiefly characterized by the predominance of extensions to the retroperitoneal and mediastinal tissues and by implantations upon the omen-

tum, mesentery, and parietal peritoneum. If death occurs in animals so affected during the first 2 months after inoculation, the extensions and implantations referred to are found to be of an extreme grade or else, and more often, some organ such as the kidneys or hypophysis is also involved. Finally, in those animals in which the disease is very mild, metastases may be found only in such sites as the eyes or suprarenal glands which do not appear to share, to an equal extent, the resistance to tumor growth possessed or developed by other tissues and organs in the body. However, secondary growths in the eyes and suprarenals also occur in cases of extreme malignancy with a widespread distribution of metastases, so that with reference to the character of the disease it is necessary to separate the instances of a mild disease from those of a severe malignancy. In addition, it should be emphasized that metastases to the skin, muscles, bones, and endocrine gland group practically never occur in cases of low malignancy.

Number and Time of Experiments.—Our first experiments served to emphasize the necessity of a larger number of animals and the desirability of a uniform time interval between operation and inoculation. We have chosen as the basis for this report the third experiment in which such factors and that of the operative technique have been satisfactorily controlled. The series was inoculated on November 14, 1924, with the thirty-ninth generation of the tumor and consisted of 10 rabbits submitted to double superior sympathectomy (14 and 15 days between operation and inoculation), 9 with double inferior sympathectomy (16, 17, and 18 days between operation and inoculation), 10 with double complete sympathectomy (9, 11, 14, and 18 days between operation and inoculation), 9 with double sympathotomy (8 and 9 days between operation and inoculation), and 10 controls.

<div align="center">RESULTS.</div>

The results of the experiment have been considered upon the basis of the general character of the disease developing in the various groups of animals rather than that of individual rabbits. The principal points are summarized in Tables I and II and Text-figs. 1 to 3. The disease of the normal or control rabbits will first be described as a means of comparison with that of the operated groups.

The primary tumor in the control rabbits grew in each instance; in two animals it was comparatively small, in two others it developed very rapidly and to extreme proportions during the first 3 weeks, while in the remainder it grew somewhat less rapidly but eventually attained a large size. The curves in Text-fig. 1 record the average calculated volume of the primary tumors of all the various groups of rabbits during the 1st month of the experiment, and it is seen that the volume in the control rabbits was somewhat greater than in the

TABLE I.

Analysis of Results.

Group No.	No. of rabbits	Mortality — Actual (per cent)	Mortality — Probable total (per cent)	Mortality — Time of death (wks.)	Incidence of paralysis (per cent)	Clinical metastases — Animal incidence (per cent)	Clinical metastases — Total No.	Clinical metastases — No. per animal	Total foci of metastases — Animal incidence (per cent)	Total foci — Total No.	Total foci — Relative rate (per cent)	Total foci — Actual rate (per cent)	No. of rabbits	No. of probable deaths	Surviving rabbits — No. of rabbits	Condition of primary tumor — Healed	Condition of primary tumor — Necrotic	Metastatic foci — No.	Metastatic foci — Relative animal incidence (per cent)	Character of disease — Severe	Character of disease — Moderate	Character of disease — Mild
1	10	40	50.	4 5 6 7 9	10	50	10	2.0	70	88	8.8	12.6	6	1	5	1	4	3	0.6	4	1	5
2	9	33	56	4 4 5 10 10	11	67	11	1.83	89	81	9.0	10.1	6	2	4	2	2	7	1.75	3	2	4

Group																						
3	10	40	60	4 4 4.5 7 9 9	10	40.	10	2.5	70	97	9.7	13.9	6	2	4	2	2	1	0.25	4	1	5
4	9	67	67	4 4 5 5.5 6 6	33	56	12	2.5	78	91	10.0	13.0	3	0	3	2	1	3	1.0	5	1	3
5	10	30	50	4.5 5 7.5 8.5 9.5	0	20	3	1.5	100	66	6.6	6.6	7	2	5	1	4	7	1.4	2	3	5

Group 1, superior; Group 2, inferior; Group 3, complete; Group 4, sympathotomy; Group 5, control.

operated animals with the exception of the sympathotomy group. Among the seven rabbits surviving at the conclusion of the experiment, the primary tumor was healed in one, largely necrotic in five, and mostly of living tissue in one instance. Three metastases were detected clinically in two rabbits. The actual mortality rate for this group was 30 per cent, the deaths occurring $4\frac{1}{2}$, 5, and $7\frac{1}{2}$ weeks after inoculation. However, it was felt, from postmortem examination at the end of the experiment, that two other animals would have eventually died from the effects of secondary tumor growths, thus making the total probable mortality 50 per cent. At autopsy one or more secondary tumors were present in each rabbit, an animal incidence of 100 per cent (Table I). The total number of metastatic foci in the group was 66, or a relative and an actual distribution rate of 6.6 per animal. A conception of the general distribution of these growths may be obtained by reference to Text-fig. 2 in which it is seen that the highest proportion of metastases occurred in the suprarenals and the eyes, while the smallest number were in the group comprising the skin, muscles, bones, and endocrine glands. As has been previously mentioned, metastases of the suprarenals and eyes usually occur either in cases of high malignancy with a widespread distribution of tumor or in instances of low malignancy in which such foci are frequently the only tumors found. There were six animals in the control group with metastases to these organs; in four the disease was mild while in two it was severe. Taking into consideration the mortality rate, the animal incidence of metastases, and the general distribution of the growths, the malignancy in the controls may be classed as of moderate or average severity.

In the four operated groups[1] the general severity of the disease or the level of malignancy was definitely higher than in the controls. The primary tumor always grew, but only in the sympathotomy group was its average volume comparable with that of the controls. In the case of the superior and complete sympathectomies the smaller size of the primary tumor was apparent. 17 days after inoculation (Text-fig. 1), while by the 3rd week the tumors of the inferior group

[1] The health and condition of the rabbits did not appear to be impaired as a result of the operations. The interval prior to inoculation was sufficient for recovery from any immediate ill effects.

were also definitely smaller than those of the controls and sympathotomy groups and continued to be so. At the conclusion of the experiment there were six surviving rabbits in the superior, inferior, and complete groups and three in the sympathotomy group. There was little difference in the character of the residual primary tumor among these groups except possibly in the case of the sympathotomy animals in which two were healed and one was practically entirely necrotic. There was one instance in each of the other three groups of a primary tumor which contained a considerable amount of living tissue. The relation of the character of the primary tumor growth to the general disease picture will be considered later.

There were more superficial metastases evident during life in all the operated groups than among the controls. Thus, after sympathotomy there were 12 in 5 rabbits (2.5 per animal), 10 in 4 after complete sympathectomy (2.5 per animal), 10 in 5 after superior sympathectomy (2.0 per animal), 11 in 6 after inferior sympathectomy (1.8 per animal), and 3 in 2 controls (1.5 per animal). As will be seen later, the most malignant disease developed in the sympathotomy and complete and superior sympathectomy groups, while the series with inferior sympathectomy fell between these and the controls. It so happens that the figures for metastases observed during life follow the same order.

The actual mortality rate (Table I) for the superior and complete sympathectomy groups, 40 per cent, was slightly higher than that for the controls, 30 per cent, while that for the inferior sympathectomies was the same, whereas the rate for the sympathotomies was more than double that of the controls, being 67 per cent. The greatest number of early deaths occurring, that is to say in the 4th and 5th weeks, occurred in the sympathotomy and complete sympathectomy groups. There was one instance in the complete, superior, and inferior sympathectomy groups and three in the sympathotomy group, of metastases to the spine causing paralysis of the hind quarters on which account the animals were killed. Among the controls there was one instance of deposits of tumor in the floor of the middle fossa of the skull beneath the dura. The total mortality rates of the several groups, which include those animals surviving throughout the experiment but which probably would have died from the effects of the tumor, approximate

each other more closely, but in the case of the sympathotomy rabbits the actual and total rates are the same, 67 per cent. There are slight differences, however, in the complete and superior sympathectomy groups which may be significant. Thus, there was an estimated mortality of six deaths in the complete sympathectomy group of which four actually occurred, and five in the superior sympathectomy groups with four actual deaths. On the other hand, among the five probable deaths in the inferior sympathectomy group and control animals, only three actually occurred.
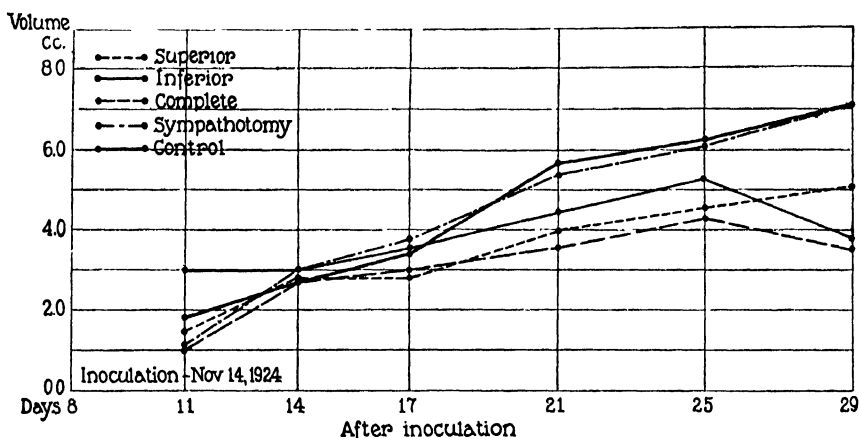
TEXT-FIG. 1. Effect of operative interference with the cervical sympathetic nervous system on the growth of the primary tumor.

With regard to the character of the disease as revealed by post-mortem examination, it is seen by reference to Table I that the highest animal incidence of metastases occurred in the controls. One or more foci of tumor tissue besides the primary tumor were found in each control animal, that is to say there was an animal incidence of 100 per cent, while the figures of the operated groups are as follows: inferior sympathectomy 89 per cent, sympathotomy 78 per cent, complete sympathectomy 70 per cent, superior sympathectomy 70 per cent. Here again, the inferior sympathectomy and control series approximate each other, while the other three operated groups fall closer together. The greatest distribution of metastases occurred in the complete sympathectomy and sympathotomy groups, the

numbers of metastatic foci being 97 and 91 respectively, while there were 88 in the superior sympathectomy, 81 in the inferior sympathectomy, with 66 in the control groups. In the sympathotomy series this distribution value may be considered as deceivingly low because of the number of what might be termed accidental deaths due to spinal metastases early in the course of the disease. Had these animals lived longer the metastatic rate would undoubtedly have been much higher and the actual amount of organ and tissue involvement considerably greater. The average number of metastatic foci per rabbit in which secondary growths occurred is as follows: complete 13.9, sympathotomy 13.0, superior 12.6, inferior 10.1, controls 6.6. The figures clearly show that upon a basis of distribution and growth of metastatic tumors the disease developing in the operated animals was considerably more malignant than among the controls, and, furthermore, that there were outspoken differences among the various operated groups which are of the same order as the mortality rate, the average number of clinical metastases per rabbit, and the total number of metastatic foci.

A more adequate conception of the general character or type of disease which developed in the several series of animals may be obtained by considering the distribution of metastatic tumors in various groups of organs according to the plan already described in the method of analysis of results.

The curves in Text-fig. 2 have been drawn upon the basis of organ and tissue grouping, the figures for the relative curves being based upon all the animals in a group, while those for the actual curves include-only those animals in which metastases were found. The actual curves parallel the relative except at one point and, in general, merely emphasize the different types of disease which actually developed. At every point in both sets of curves, except in the relative curve in the case of the eyes and suprarenals of the superior sympathectomy group, the incidence and distribution of metastases are seen to be greater in the operated than in the control animals. The sympathotomy and inferior sympathectomy curves are similar in form, but the former is consistently higher and is considerably higher in the last column which records the involvement of the skin, muscles, bones
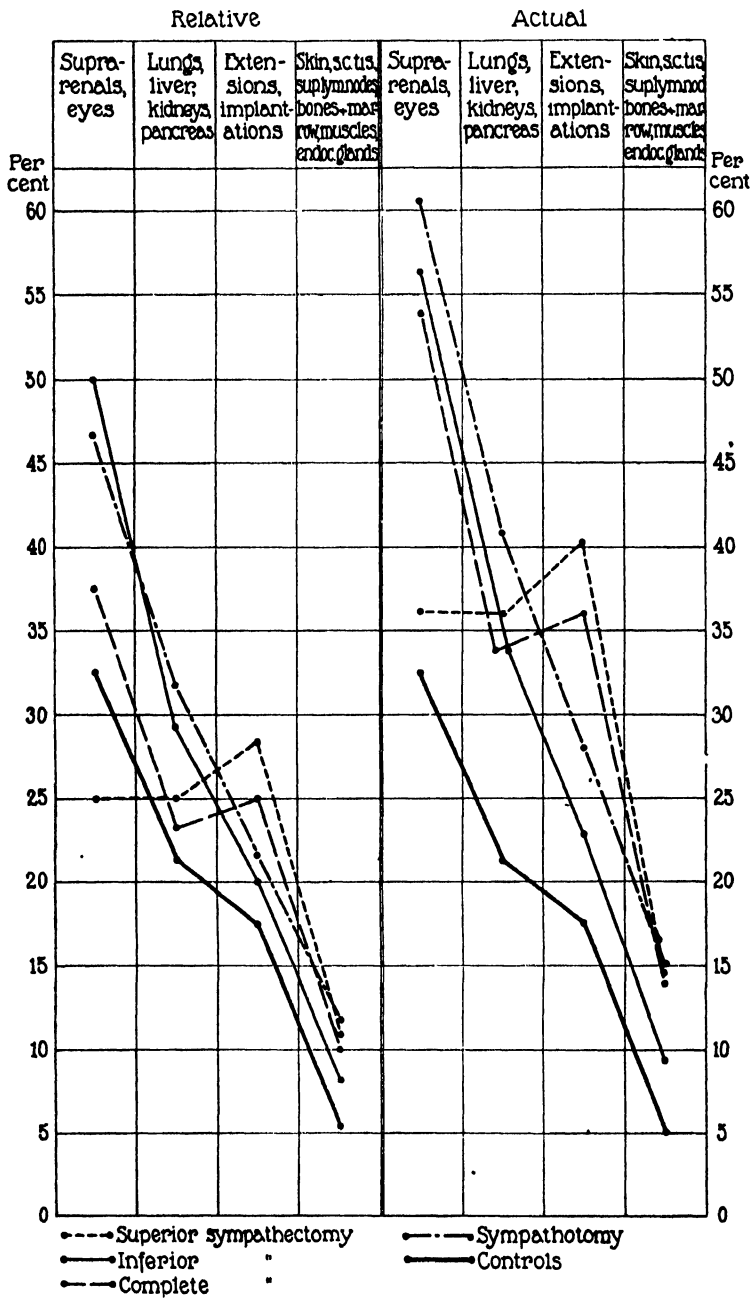
TEXT-FIG. 2. Distribution of metastatic foci.

etc.[2]   The curve representing metastases in the complete sympathec-
tomy rabbits is almost the same as that of the inferior sympathectomy
group in the first two columns (suprarenals and eyes; lungs, liver,
kidneys, pancreas), but is much higher as regards extensions and
implantations and definitely higher as regards skin, muscles, bones,
and endocrine glands.   The curve for the superior sympathectomy
group resembles that of the complete sympathectomy animals, except
in the case of suprarenal and eye metastases, in which it approaches
the control curve.[3]

Upon the basis of these two sets of curves, one would conclude that
the disease which developed in the four operated groups was on a much
higher plane of malignancy than that of the control animals and,
further, that the disease of greatest severity occurred in the sympathot-
omy and complete sympathectomy rabbits.   In order to classify the
inferior and superior sympathectomy series, however, an additional
analysis is needed of the type of disease in which metastases occurred
to the suprarenals and eyes.   Relatively little importance has been
attached to the incidence of extensions and implantations, but the
fact that the figures for the superior sympathetic series are definitely
greater than those for the inferior sympathetic animals in the groups
of parenchymatous organs and the skin, muscles, bone, etc., indicates
that the much greater value of the inferior sympathetic series for
suprarenal and eye metastases is indicative of a mild or chronic disease
rather than of greatly increased malignancy.

[2] Differences in the figures for the skin, muscle, bone, and endocrine gland group
which appear comparatively small are actually of much greater significance
than in the case of the other subdivisions because of the larger number of organs
and tissues making up the group, the total figure for which is used in arriving at
the percentage estimation values (6).

[3] While the level of malignancy of this superior sympathectomy group was much
higher than that of the control animals, one of our preliminary experiments sug-
gests that such may not always be the case.   In this experiment there were only
five superior sympathectomized rabbits, but the general character of the disease
for the group was only slightly more severe than that of the controls.   A possible
explanation of this result may lie in the much longer interval between operation
and inoculation—6 weeks—which may have been sufficient for such a readjust-
ment of the animal organism that its resistance capacity reached the level for
normal animals prevailing at that time.

The significance of the growth in the suprarenals and eyes is brought out in Table II which summarizes the principal points regarding the relation of these metastases to the total number of metastatic foci occurring in these rabbits as well as in all the animals of each group. The highest animal incidence and the largest number of suprarenal and eye tumors occurred in the inferior and sympathotomy series, but the inferior and control groups showed the greatest proportion of these growths as compared with the total number of metastatic foci of these rabbits (22.5 per cent), with the sympathotomy, complete,

TABLE II.

Analysis of Metastases to Suprarenals and Eyes.

Group No.	No. of rabbits.	Proportion of rabbits with suprarenal and eye metastases to all rabbits with metastases.	Suprarenal and eye metastases.			Total No. of metastatic foci in entire group.	Proportion of suprarenal and eye metastases to metastatic foci of entire group.		Type of disease.			Proportion of mild types of disease.
			Total No.	Total No. of metastatic foci in these rabbits.	Proportion to total No.		Relative.	Actual.	Severe.	Moderate.	Mild.	
		per cent			*per cent*		*per cent*	*per cent*				*per cent*
1	4	59.1	10	68	14.7	88	25.0	35.7	3	1	0	0
2	7	87.5	18	80	22.5	81	50.0	56.3	3	1	3	42.8
3	6	85.7	15	96	15.6	97	37.5	53.6	.4	1	1	16.6
4	7	100.0	17	91	18.8	91	47.2	60.7	5	1	1	14.2
5	6	60.0	13	58	22.5	66	32.5	32.5	2	0	4	66.7

Group 1, superior; Group 2, inferior; Group 3, complete; Group 4, sympathotomy; Group 5, control.

and superior groups following in the order named (18.8, 15.6, 14.7 per cent). Furthermore, when one classifies the type of disease which developed in the rabbits in which suprarenal and eye tumors were found, it is seen that the largest number of mild cases occurred in the control and inferior sympathectomy animals, 67 and 43 per cent respectively, while there were only 17 and 14 per cent of mild cases in the complete sympathectomy and sympathotomy groups, and none in the superior sympathectomy group. These points are graphically illustrated by a second set of curves from which the metastases to the suprarenals and eyes occurring in cases of low malignancy have
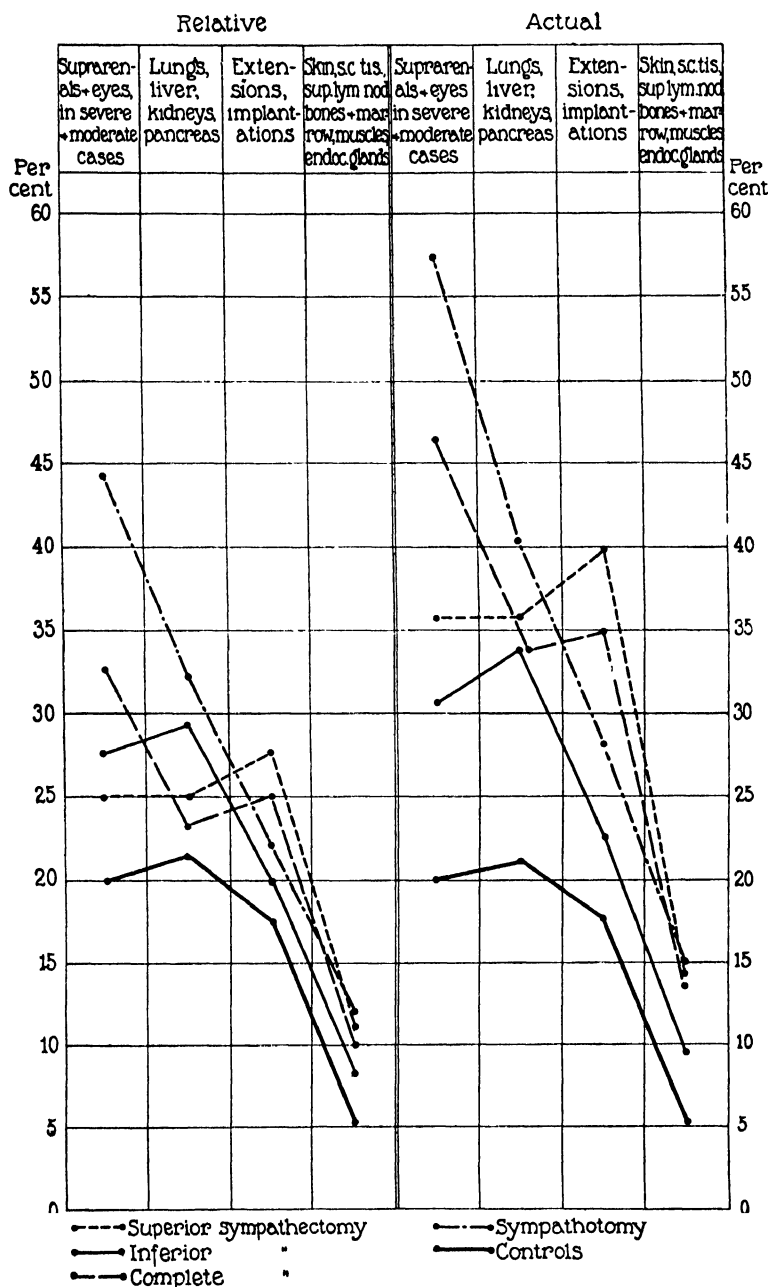
TEXT-FIG. 3. Distribution of metastatic foci omitting those to the suprarenals and eyes occurring in mild cases.

been omitted (Text-fig. 3), giving a more adequate conception of the precise character of the disease of the various groups. The curves representing the operated series are seen to be markedly higher than that of the controls. There is not a great deal of difference between the curves for the superior and complete sympathectomy groups, the former being slightly higher, except in the case of metastases to the eyes and suprarenals, but the disease of the inferior sympathectomy group is now clearly shown to be the least malignant of any occurring in the operated series. The order of malignancy of the several groups, as shown by these curves, is therefore as follows: sympathotomy, superior and complete sympathectomy, inferior sympathectomy, controls.

Finally, the condition of those rabbits surviving the experiment and not considered as probable ultimate deaths should be mentioned. The number of such animals is practically the same for all groups, four or five (Table I), except in the case of the sympathotomy series, in which there were three. A few foci of metastases were found at autopsy, from one to seven per group, and the relative animal incidence of these growths again brings out the resemblance of the inferior sympathectomy group to the controls (1.75 and 1.4 foci per animal), while the sympathotomy, the superior and complete sympathectomy groups are associated together on a plane of higher malignancy (1.0, 0.6, and 0.25 per animal).

<center>DISCUSSION.</center>

It is evident from this brief analysis that the various operations performed upon the cervical sympathetic ganglia and nerves brought about a condition of the rabbit host which permitted the development in the several groups of animals of a disease considerably more malignant than that prevailing among controls inoculated at the same time. Furthermore, there were differences in the malignancy level among the groups themselves. Upon a basis of actual and total probable mortality, the time of actual deaths, the number and animal incidence rate of clinical metastases, the total number of metastatic foci, and finally the general character of the disease as revealed by the distribution of these metastases, the order of malignancy in the operated groups beginning with that manifesting the most severe disease was as follows: sympathotomy, complete sympathectomy, superior sympathectomy, inferior sympathectomy, controls.

There were two points in which the disease of the controls might be considered as more malignant than in the operated animals. The average volume of the primary tumor was greater for the control group during the 1st month than that of any of the operated groups except the sympathotomy. It has been pointed out, however, that in a disease of a fairly high malignant level animals with the largest primary tumors are likely to show a less severe metastatic involvement than those with smaller primary growths (7). This generalization does not hold for a disease of a more severe character and consequently cannot be applied to the operated groups.

The rate for the animal incidence of metastases in the controls was 100 per cent, while that of the operated groups ranged from 70 to 89 per cent. In other words, tumor tissue in addition to the primary tumor was found in all the control animals, while two or three rabbits in each operated group had been able completely to prevent or suppress any metastatic growths. Although there was only a single focus of secondary tumor in four control rabbits, consisting of a cord nodule or a metastasis to one suprarenal gland, neither of which would have caused the death of the animal, still such a finding indicates that in certain rabbits or under certain conditions the surgical procedures employed did not invariably induce a state of decreased resistance to the tumor.

We have drawn attention to the fact that in any group of five or ten normal rabbits the proportion of those with high, low, and intermediate grades of resistance to this tumor, and to infections with *Treponema pallidum* as well, is roughly 1:1:3. The operative procedures employed in this experiment have not disturbed this ratio as regards the proportion of animals with high resistance, but apparently has done so in the case of the intermediate or low grades, since instances of moderate and severe malignancy of each group were considerably more numerous than in similar cases in the controls.

The interpretation of the facts that have been presented with regard to the general character of this malignant disease in rabbits after operative removal of various portions of the cervical sympathetic nerves can be only tentative, since our knowledge of the anatomy and physiology of these structures in the rabbit is slight. In particular there is no definite information available upon the connection between

the cervical sympathetic and the thyroid gland, though from what is known in the case of man and certain of the lower animals, it is probable that a relation of some sort exists. Our results furnish some evidence that both the superior and inferior ganglia are important structures in the mechanism of animal resistance to an induced malignant disease, the superior ganglia appearing to be the more essential in this relation. Whether it operates through the thyroid or by some other means is not certain, but the effects of complete thyroidectomy and of removal of the superior cervical ganglia, or of a portion of the sympathetic nerves, are, on the whole, strikingly similar in terms of general malignancy. There is in both cases a clear-cut increase in the severity of the disease. But, while removal of the thyroid practically always induces this effect, there are certain rabbits in which superior sympathectomy or sympathotomy did not. Furthermore, the rapid and very extensive growth of the primary tumors characteristic of completely thyroidectomized rabbits is not seen in superior sympathectomized animals nor is it equalled in the sympathotomized animals.

Removal of the inferior cervical ganglia resulted in a less malignant disease than that which developed in the superior and completely sympathectomized and the sympathotomized groups but one more severe than in the controls.

If the superior ganglia are the more important or essential elements in the cervical sympathetic system, then their removal alone or as a part of a complete cervical sympathectomy should bring about analogous effects. Such was apparently the case in this experiment. On the other hand, removal of a small portion of the cervical sympathetic nerve alone, without surgical interference of the superior or inferior ganglia, brought about a similar but even greater effect than removal of the superior ganglia or of the entire system. One might presume that extirpation of the inferior ganglia, in which the nerve is necessarily sectioned as in the sympathotomy operation, might bring about a similar effect. This did not occur, however.

The reasons for these various states of malignancy, or conversely, of animal resistance are not clear. The results of the experiment suggest several explanations as possible. The character of the malignant disease may be considered as a result of two major factors, (1) the

growth of the inoculated tumor cells and (2) the defensive forces which the animal host brings to play against these cells. There was a good initial growth of the primary tumor in every animal and we can assume that the operative procedures themselves did not interfere with or favor, to any material extent, the initial stage of the disease. On the other hand, since the eventual character of the disease as a whole was more malignant in the operated than in the normal animals, it is evident that the natural or acquired defensive forces of the host were in some way rendered less effective in the former.

When the superior ganglia were removed, either alone or as a part of a complete extirpation of the cervical system, or a medial portion of the nerves was removed, the mechanism of resistance was less efficient than when the inferior ganglia alone were extirpated. In both ganglion operations the nerve was necessarily cut, but one set of ganglia was not disturbed. It would seem, therefore, that these structures are associated with more than a single functional effect. There is probably a coordinating function of some kind between the two ganglia; yet, in addition, it would seem that both a favorable and a deleterious influence is associated with them. Thus, when the superior ganglia alone are extirpated a markedly unfavorable effect predominates; when the inferior ganglia alone are removed a much less deleterious effect is observed; whereas when both superior and inferior ganglia and the connecting nerves are extirpated the unfavorable effect is similar to that observed after removal of the superior ganglia. In other words, the absence of the inferiors does not increase or decrease the deleterious effect produced by the removal of the superior ganglia, so that it would seem that whatever unfavorable effect is produced by an inferior sympathectomy is mainly caused by an interruption of the path and hence of the coordinating function existing between the superior and inferior ganglia and that the additional removal of the inferior ganglia permits of the development of a favorable influence. Such an influence is not appreciable when the path between the ganglia is simply interrupted and the inferior as well as the superior ganglia are undisturbed. When this is the case, an outspoken deleterious effect upon resistance is to be seen, as witness, the severe character of the neoplastic disease. Under these conditions, the presence of either the superior or the inferior ganglia would appear to be a disadvantage.

If the effect of the various surgical procedures is obtained through the thyroid gland, the experiments would suggest that the absence of the superior cervical ganglia is associated with a condition of lowered or abolished thyroid function, as in the case of completely thyroidectomized rabbits in which there is a relatively ineffective resistance to the tumor.   Furthermore, it would appear that since the absence of the inferior ganglia alone is associated with a less pronounced decrease in animal resistance the intact superior ganglia are still capable of exerting a favorable influence or of counteracting the unfavorable one brought about by the removal of the inferiors.   However, since a distinct deleterious effect upon animal resistance followed an interruption of the direct nerve pathway between the undisturbed sets of ganglia, it is obvious that other factors are involved than are apparently associated with a simple extirpation of the ganglia.

Whatever may be the ultimate explanation of these apparently paradoxical results, it is clear, from this experiment, that removal of the cervical sympathetic system or certain of its component parts is, in the rabbit, followed in many instances by a less effective resistance to a transplantable neoplasm.   In consequence, the character of the disease which develops in such animals is highly malignant.

SUMMARY AND CONCLUSION.

The effect of the removal of the complete cervical sympathetic nervous system, of both superior and of both inferior cervical ganglia, and of a small portion of the cervical sympathetic nerve in the rabbit was studied in relation to the character of a malignant disease induced by a transplantable neoplasm.

It was found that the general character of the disease which developed in the operated groups of animals was more severe than that of a similar sized control group.   Comparisons of the mortality rate in the several groups, of the animal incidence of metastases, the number of metastatic foci, and the distribution of these secondary growths all showed this to be the case.   There appeared, furthermore, to be differences in malignancy among the operated groups themselves.   The most severe disease occurred in the group in which a portion of the sympathetic nerve only was removed (sympathotomy); that in the

complete sympathectomy and superior sympathectomy groups was slightly less malignant; and that in the inferior group was much less so.

These results have been interpreted as due to a less effective animal resistance, the mechanism of which has been interfered with in some way by the interference with the sympathetic nerves. The reasons for the difference in malignancy exhibited by the several operated groups are undetermined. A tentative explanation is suggested upon the basis of coordinating, favorable or deleterious functions subserved by the cervical sympathetic nervous system.

BIBLIOGRAPHY.

1. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 472.
2. Brown, W. H., Pearce, L., and Van Allen, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 373.
3. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 476.
4. Pearce, L., and Van Allen, C. M., *Tr. Assn. Am. Phys.*, 1923, xxxviii, 315.
5. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 601, 631, 799, 811. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 583.
6 Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1923, xxxviii, 347.
7. Van Allen, C. M., *J. Exp. Med.*, 1925, xli, 691.

BIOLOGY OF BACTERIUM LEPISEPTICUM.

II. The Structure of Some Iron Compounds Which Influence the Growth of Certain Bacteria of the Hemophilic, Anaerobic, and Hemorrhagic Septicemia Groups.

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In a previous report, one of us has shown that certain virulent cultures of *Bacterium lepisepticum* need, for optimum growth and maintenance, either a plain nutrient medium at a reduced oxygen tension or, under aerobic conditions, this same medium plus rabbit blood.[1] The blood was shown to function in high dilutions to the limit of the benzidine reaction, and also after autoclaving for 30 minutes at 115 pounds pressure. For these reasons it was concluded that the active substance contained in blood could not be a food, nor of vitamine nature. But its behavior did suggest a function similar to that of the X factor, the presence of which Avery and his associates[2] regard as one of the essentials for optimum growth of hemophils, anaerobes, and pneumococci at atmospheric pressure.

Avery has described the X factor as follows: "The so called X factor, which is associated with the pigment fraction of blood, gives the peroxidase reaction, and is not destroyed by moist heat at 120°C. . . . . Moreover, the X substance is active in such minute amounts as to suggest that it functions as a biocatalyst."[3] It would seem then that the principle needed for good growth of virulent strains of *Bacterium lepisepticum* resembles the X factor.

The X factor has been found in plant tissue[2] and in a solution of ferrous sulfate and gum arabic.[4] A list of substances which function as X has been assembled

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[1] Webster, L. T., *J. Exp. Med.*, 1925, xli, 571.

[2] Avery, O. T., *et al.*, *J. Exp. Med.*, 1921–24, xxxiv, xxxviii–xl.

[3] Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, xxxix, 296.

[4] Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 59.

from the literature by Novy.[5]   The chemical constitution of most of them is however, so little understood that it seemed advisable to test the action of simple compounds in the hope that additional facts relating to the structure and behavior of the X mechanism might be recognized.

Technique.

A number of substances with some of the properties of hemoglobin have been used; sodium pentacyano-aquo-ferroate and its derivatives, ferrous and ferric oxides, and vegetable charcoal.

The peroxidase reaction was carried out with either benzidine chloride dissolved in distilled water, or benzidine dissolved in glacial acetic acid, plus superoxol (hydrogen peroxide, Merck).   The presence of ferrous iron was detected by the Prussian blue reaction, or by titration with $KMnO_4$.   The amount of oxygen absorbed by a compound was determined by means of a Barcroft-Haldane apparatus as modified by Brodie.[6]

The biological test consisted in adding the given substance to tubes containing 5 cc. of plain meat extract broth, pH 7.4, and inoculating each with *Bacterium, lepisepticum*, Type D, in numbers varying exponentially from 10^9 to 10^0.   If growth occurred in tubes seeded with less than 100,000 bacteria, and if, after 3 or more weeks incubation at $37°C.$, no Type G variants appeared in any culture, the test was considered positive and the compound was considered to function as the X factor.[1]

Sodium Pentacyano-Aquo-Ferroate.

Baudisch's studies of the catalytic properties of iron[7] led us to test the activity of sodium pentacyano-aquo-ferroate, an inorganic compound of known formula discovered by Hofmann.[8]   This substance, like hemoglobin, contains a ferrous radical, gives a strong peroxidase reaction, and absorbs oxygen at $37.5°C.$, at an initial rate of 2.38 cc. per gm. per hour.[9]   It is derived from the inactive sodium ferrocyanide by the replacement of one NaCN group by H_2O.   This H_2O group,

[5] Novy, F. G., Jr., *J. Infect. Dis.*, 1925, xxxvi, 343.

[6] Brodie, T. G., *J. Physiol.*, 1909–10, xxxix, 391.

[7] Baudisch, O., and Welo, L. A., *J. Biol. Chem.*, 1924, lxi, 261.

[8] Hofmann, K. A., *Ann. Chem.*, 1900, i, 312.

[9] We are indebted to Dr. James A. Hawkins for measuring the oxygen absorption of these compounds.   His readings for sodium pentacyano-aquo-ferroate were as follows:

Cell 14 = −2.720 cc. O_2 consumed per gm. per hour.
" 23 = −2.010 " O_2 " " " " "
" 13 = −2.41 " O_2 " " " " "

although forming an integral part of the molecule, is associated with an available bond which endows the central iron atom with activity, catalase, oxygenase, and peroxidase properties.

Experiment 1.—Nine tubes containing 5 cc. of plain extract broth, pH 7.4, plus 1 cc. of a 10 per cent sterile solution of the aquo salt, nine tubes of broth plus 1 cc. physiological salt solution, and nine tubes of broth plus 1 cc. of a 10 per cent solution of autoclaved, laked rabbit corpuscles were inoculated with a 17 hour broth culture of *Bacterium lepisepticum*, Type D, in dilutions increasing exponentially from 1 to 9. After 24, 48, and 72 hours incubation the cultures were examined for growth and later for the appearance of Type G variants.

The results are shown in Table I. Growth occurred in all the aquo salt and blood tubes, including those which had received the smallest

TABLE I.

Effect of Sodium Pentacyano-Aquo-Ferroate on the Growth of Bacterium lepisepticum, Type D.

Medium.	Benzidine test	Reducing power.	Oxygen-absorbing power.	No. of bacteria per tube.									Type G variation.
				10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}	
Plain broth..........	0			++	++	++	0	0	0	0	0	0	++
Plain broth + laked blood corpuscles....	++	++	++	++	++	++	++	++	++	++	++	++	±
Plain broth + aquo salt...............	++	++	++	++	++	++	++	++	++	++	++	++	±

0, negative or no growth; ±, occasional variant after 3 weeks; ++, positive, good growth, many variants in 48 hours.

number of organisms, but only in such of the plain broth tubes as had been seeded with 100,000 or more. Later, Type G variants appeared in these cultures, but not in the blood or aquo salt tubes.

Further experiments demonstrated that this aquo salt was active as the X factor in dilutions of 1/100,000 gm. per cc. This dilution was also the limit of the benzidine reaction. The ferric salt was found to be active as well, but the presence of NO_2 and NH_3 groups in place of H_2O decreased its sensitivity.

Experiments with strains of *Bacillus avisepticus* yielded similar results.
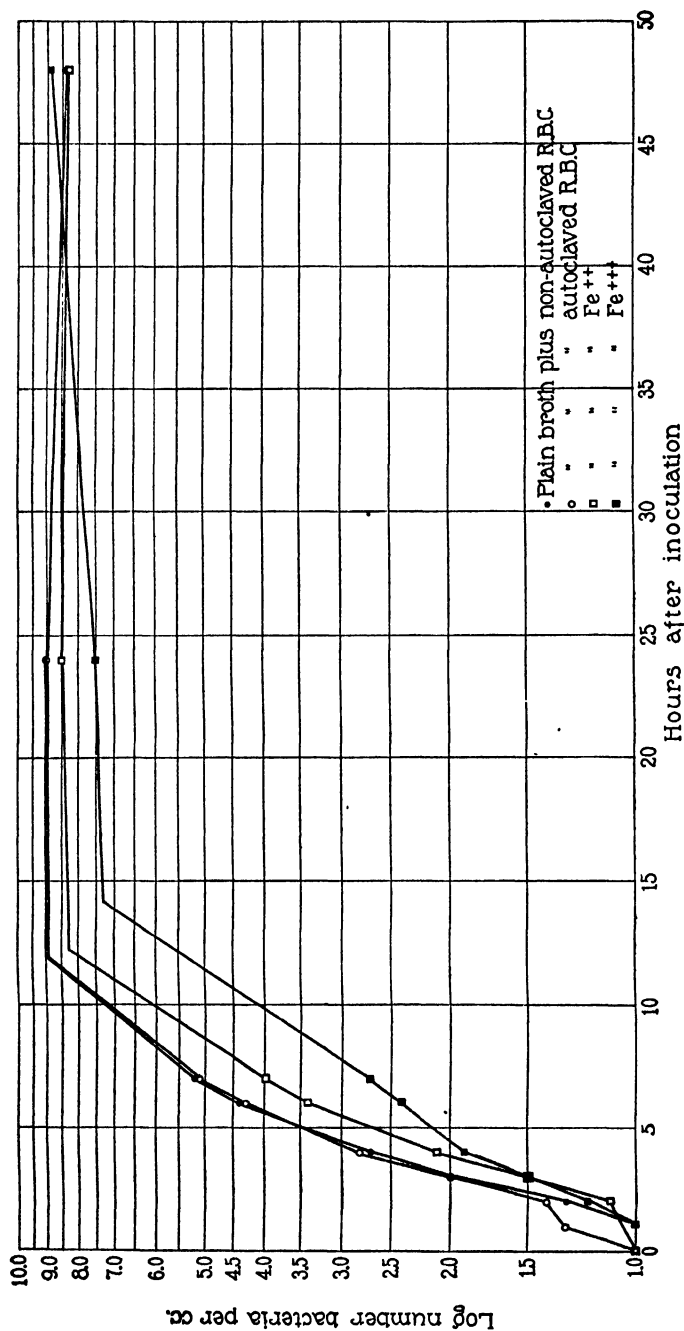
TEXT-FIG. 1.   Growth of *Bacterium lepisepticum*, Type D, in plain broth plus aquo salt.

In the work which followed a determination of the rate of bacterial growth was made by the serial counting method.[1]

Experiment 2.—To one Erlenmeyer flask containing 150 cc. plain broth was added 18 cc. hemolyzed, washed rabbit blood corpuscles plus 12 cc. 0.85 per cent NaCl; to another, 20 cc. autoclaved red cells plus 10 cc. saline; to a third, 30 cc. of an aqueous 10 per cent solution of the aquo ferrous salt, and to a fourth, 30 cc. of the aquo ferric salt. Samples from the first three flasks gave strong benzidine reactions; in Flask 4 the test was weaker.

Each flask was now inoculated with 1 cc. of a 1:1,000,000 dilution of a 17 hour plain broth culture of *Bacterium lepisepticum*, Type D, and incubated at 37°C. Counts were made at half hour intervals for the first 6 hours; then at 24 and 48 hours. The numbers of bacteria per cc. in each flask at various time intervals are plotted in Text-fig. 1.

Following an inoculation of less than 10 bacteria per cc., the count in each flask increased immediately and logarithmically to reach a maximum of about 1 billion per cc. within 24 hours. The growth curves of the bacteria in the flasks containing broth and blood, and broth and aquo ferro salt paralleled one another closely; that from the ferric salt medium differed in that the rise was somewhat less abrupt. Evidently the aquo salt, by supplying the X factor, furnishes optimum conditions for the aerobic growth of *Bacterium lepisepticum*.

Experiment 3.—This experiment was planned to test the activity of the aquo ferrous salt as the X factor in the growth of organisms of the so called hemophilic group.

Three cultures were obtained from Dr. T. M. Rivers. The first, *B. influenzæ* No. 6,[10] required for growth the presence of the X and V factors; the second, "J. R. L.,"[11] needed the V factor alone; and the third, "Dog,"[12] the X factor only. These strains were taken from blood agar slants and inoculated into 10 per cent rabbit blood broth tubes. After 24 hours incubation, 0.1 cc. from each tube was transferred to a tube containing 4 cc. of plain broth, pH 7.4, plus 1 cc. of a 10 per cent solution of the aquo salt. The media gave a strong benzidine test prior to inoculation. These first subcultures were then incubated overnight and on the following day 0.1 cc. was plated on chocolate agar to determine the presence of live organisms, and one loopful was transferred to the second series of aquo salt broth tubes.

This procedure was continued for six subcultures, each after an incubation period of 24 to 48 hours. Then 0.1 cc. of each culture was transferred to tubes of

[10] Rivers, T. M., and Poole, A. K., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 202.

[11] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 429.

[12] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 149.

TABLE II.

Growth of Influenza Bacilli in Media Containing Aquo Salt.

Culture.	Subculture No.	Aquo salt.	Control plates.	Aquo salt + yeast.	Subculture No.	Control plates.	Yeast.	Subculture No.	Control plates.	Blood.	Control plates.	Plain broth.
Infl.										++	++	0
J. R. L.										++	++	0
Dog.										++	++	0
Infl.	1	±	+									
J. R. L.	1	±	+									
Dog.	1	++	++									
Infl.	2	±	+									
J. R. L.	2	±	+									
Dog.	2	++	++									
Infl.	3	±	+									
J. R. L.	3	±	+									
Dog.	3	++	++									
Infl.	4	±	+									
J. R. L.	4	±	+									
Dog.	4	++	++									
Infl.	5	±	+									
J. R. L.	5	±	+									
Dog.	5	++	++									
Infl.	6	±	+									
J. R. L.	6	±	+									
Dog.	6	++	++									
Infl.	7	±	+	++	1	++	++	1	++	++	++	0
J. R. L.	7	±	+	++	1	++	++	1	++	++	++	0
Dog.	7	++	++	++	1	++	++	1	++	++	++	0
Infl.	8	±	+	++	2	++	0	2	0	++	++	0
J. R. L.	8	++	++	++	2	++	++	2	++	++	++	0
Dog.	8	++	++	++	2	++	±	2	+	++	++	0
Infl.	9	±	+	++	3	++	0	3	0	++	++	0
J. R. L.	9	±	+	++	3	++	++	3	++	++	++	0
Dog.	9	++	++	++	3	++	0	3	0	++	++	0

±, survival, very slight growth; +, moderate growth; ++, good growth.

aquo salt broth, aquo salt plus 1.0 per cent yeast extract broth, and 1.0 per cent yeast extract broth. Blood and plain broth tubes served as controls. Plates were smeared the next day and subcultures were made from each tube to a second series of similar media. Three consecutive transfers were carried out in this manner.

Table II shows the results. The Dog culture grew well in aquo salt broth, but *Bacillus influenzæ* and J.R.L., although surviving for nine subcultures, grew very poorly. All three cultures grew profusely in broth containing aquo salt plus yeast extract, and only J.R.L. grew in the media containing yeast extract alone. From these experiments it was concluded that the aquo salt functions as the X factor in the growth of *Bacillus influenzæ* and other hemophils as well as in the case of bacteria of the hemorrhagic septicemia group.

Strains of *Bacillus welchii* and *Bacillus sporogenes* which failed to grow in tubes of aerobic broth sealed with vaseline, but which did multiply luxuriantly when the oxygen tension in the broth was reduced mechanically by boiling and sealing, showed similar abundant growth when aquo salt was added to aerobic broth sealed with vaseline.

Further experiments were made with oxides of iron which were being studied by Welo and Baudisch.[13] Fe_3O_4, natural magnetite, was first tried and, although quite insoluble, was found to give a positive benzidine reaction and to function as the X principle for the growth of *Bacterium lepisepticum*.

Iron Oxides.

Fe_3O_4, Lefort's oxide, was then made by precipitating one part $FeSO_4$ and one part $Fe_2(SO_4)_3$ with NaOH. This substance possesses a definite cubic crystal structure, a magnetic susceptibility of 0.152, absorbs oxygen at an initial rate of 0.0043 cc. per gm. per hour,[14]

[13] Welo, L. A., and Baudisch, O., *J. Biol. Chem.*, 1925, lxv, 215.
[14] Dr. Hawkins' readings for the oxides were the following.

Fe_3O_4 Cell 14 = 0.0045 cc. O_2 consumed per gm. per hour.
      " 23 = 0.0043 " O_2 "   "   "   "   "
      " 13 = 0.0044 " O_2 "   "   "   "   "
Fe_2O_3 active Cell   14 = 0.0072 cc. O_2 consumed per gm. per hour.
      "   23 = 0.0092 " O_2 "   "   "   "   "
      "   13 = 0.0076 " O_2 "   "   "   "   "
Fe_2O_3 inactive Cell 14 = 0
      "   23 = 0
      "   13 = 0

and gives a positive benzidine reaction in broth. Oxidation at 330° removed all trace of ferrous iron, but did not affect the crystal structure. The magnetic susceptibility and initial rate of oxygen absorp-

TABLE III.

Effect of Ferric Oxides on the Growth of Bacterium lepisepticum, Type D.

Medium.	Benzidine.	Ferrous iron.	Initial rate of oxygen absorption.	No. of bacteria per tube.									Type G variants.
				10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}	
			cc. per gm. per hr.										
Plain broth....	0	0	0	++	++	++	++*	0	0	0	0	0	++
Plain broth + blood......	++	++	—	++	++	++	++	++	++	++	++	++	±
Plain broth + Fe₃O₄.......	++	++	0.0043	++	++	++	++	++	++	++	++*	0	±
Plain broth+ active Fe₂O₃.	++	0	0.008	++	++	++	++	++	++	++	+*	0	±
Plain broth+ inactive Fe₂O₃.......	0	0	0	++	++	++	++*	0	0	0	0	0	++

—, no determination.

* Growth after 48 hours incubation.

0, negative or no growth; ±, occasional variant after 3 weeks; ++, positive, good growth, many variants in 48 hours.

tion increased slightly.[14] The benzidine test continued positive. Further heating to 550° changed the crystal structure unit to a rhombohedral form, destroyed its magnetic and oxygen-absorbing properties,[14] and its power to oxidize benzidine. Catalase activity was present before and after heating.

The biological test was carried out on these three oxides as follows:

Experiment 4.—About 0.5 gm. of each of these substances, Lefort's oxide (Fe_3O_4), active Fe_2O_3, and inactive Fe_2O_3, was added to each of nine tubes containing 5 cc. of plain broth, pH 7.4. These three series together with a control set of plain broth and blood broth tubes were inoculated with a 17 hour plain broth culture of *Bacterium lepisepticum* in dilutions increasing exponentially from 1 to 9. After 24, 48, and 72 hours incubation they were examined for growth. Later the variant G forms were sought for by plating methods.

Table III shows the results of this experiment.   Growth occurred in every tube to which blood, Fe_3O_4, or active Fe_2O_3 had been added, and no G variants appeared for 2 weeks.   In the plain broth and inactive Fe_2O_3 series bacterial multiplication occurred only in those tubes which had received an inoculation of 100,000 organisms or more. G forms were abundant in these after 72 hours.   Further quantitative tests showed that at least 0.1 gm. of the active oxides per cc. of broth was necessary for a positive reaction.

Bacillus avisepticus behaved in a similar manner.   The hemophilic and anaerobic strains used in the previous experiments grew well in the presence of the active Fe_2O_3, while no growth occurred when the inactive oxide was used.

DISCUSSION.

Two types of inorganic iron compounds of known structure have been found to show the so called X type of biocatalytic activity, (1) pentacyano iron salts and (2) certain iron oxides.   In each instance definite physical and chemical properties may be related to the growth-promoting power.   Sodium pentacyano-aquo-ferroate contains an unsaturated bond which is associated with oxygen-absorbing and peroxidase properties, as well as with growth-promoting activity.   And Fe_3O_4, although quite insoluble, has similar characteristics.   When this latter substance is heated just enough to oxidize all ferrous iron, a form of Fe_2O_3 is obtained which retains the same crystal structure, oxygen-absorbing, peroxidase, and growth-activating properties. Further heating of this Fe_2O_3 alters its crystal structure and destroys its oxygenase, peroxidase, and biocatalytic action.

The contrast in growth-promoting power of these two similar iron oxides indicates anew the close association of this property with that

of oxygen absorption and of benzidine oxidation. Avery regards the iron-containing X substances as peroxidases which destroy metabolic, toxic, organic peroxides, and which, in some more subtle way, activate cell respiration. Novy, on the other hand, considers that the mechanism of all chemical, animal, and vegetable X substances is to reduce the oxygen tension of the medium to a level suitable for growth.[5]

The data so far accumulated indicate that, though many X substances have characteristics in common with respiratory ferment, the only property common to all is that of being able to absorb oxygen.

The effect of these biocatalysts on the appearance of bacterial variants should be noted. Their presence in the media insures optimum growth and inhibits variation, while without them growth is retarded and variants are abundant after 48 hours. Apparently the occurrence of this type of bacterial variation is intimately associated with the presence of excess oxygen in the medium.

<div align="center">CONCLUSIONS.</div>

Inorganic iron substances of known chemical structure have been tested for their ability to function as the X factor in the growth of bacteria of the hemophilic, anaerobic, and hemorrhagic septicemia groups.

Each iron compound possessing this biocatalytic activity showed oxygen-absorbing and peroxidase properties.

The interrelation of these mechanisms is discussed.

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

III. SOME OF THE FACTORS DETERMINING THE NUMBER AND SIZE OF PLAQUES OF BACTERIAL LYSIS ON AGAR.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 20.

The quantitative evaluation of the activity of the so called bacteriophage is usually accomplished by one of two methods.

The earlier procedure and its modifications[1,2] make use of the appearance of clear spots—"*taches vierges*" or "plaques"—in the midst of an even growth when bacteria are seeded on agar in the presence of bacteriophage-containing solutions. It is assumed that each clear spot represents a colony of "bacteriophage," and the count of these colonies is carried out in a manner analogous to that used in counting colonies of bacteria.

The other method[3,4] consists in preliminary serial dilution of the bacteriophage-containing fluid in sterile broth, with subsequent inoculation of these serial dilutions with a standard suspension of susceptible bacteria. In this case it is assumed that the highest dilution in which the lysis of bacteria occurs after a proper incubation must have received at least one unit of lytic principle and thus the total number of active units present in a given volume of the original fluid may be computed.

If in the case of "bacteriophage" one is dealing with a suspension of discrete living elements, as assumed by d'Hérelle, the count of these particles by both methods should be consistent, as it is when used in enumerating bacteria, and therefore the methods could be used inter-

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[1] d'Hérelle, F., Le bactériophage, son rôle dans l'immunité, Monographies de l'Institut Pasteur, Paris, 1921, 17.

[2] Gildemeister, E., and Herzberg, K., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 12.

[3] Appelmans, R., *Compt. rend. Soc. biol.*, 1921, lxxxv, 1098.

[4] Werthemann, A., *Arch. Hyg.*, 1922, xci, 255.

changeably. However, the observations of several investigators,[5-8] as well as our own experience,[9] have shown that the lytic titers of bacteriophage-containing fluids are considerably higher when determined by the broth dilution method than on counting the plaques on agar. Before going into the reason for this discrepancy, we have felt that it is necessary to establish first whether the lytic principle is really particulate, as assumed by d'Hérelle, or whether, on the contrary, it is present in a state of true solution.

Particulate Distribution of the Lytic Principle.

When a broth culture of young, susceptible bacteria is allowed to grow in the presence of suitable amounts of the active lytic principle, the concentration of the latter can be shown to increase rapidly during the period of logarithmic growth of bacteria. After 18 to 24 hours of incubation the concentration of the lytic principle usually reaches its highest level, after which it remains unchanged, irrespective of further changes in the bacterial count. If at the time bacteria are removed by filtration, the filtrate is distributed in a series of broth tubes in gradually diminishing amounts, from 0.1 cc. down, and if each of the tubes of the series is seeded with 0.1 cc. of a suspension of young culture (18 hours) containing about 1,000,000,000 of susceptible bacteria per cc., one finds that after a suitable period of time (18 to 24 hours) lysis of bacteria has taken place in all tubes receiving more than 10^{-10} cc. of the filtrate. All the tubes receiving less than 10^{-10} cc., with but few exceptions, show no lysis. Moreover, if the tubes are incubated further for 18 to 24 hours, and if at this time their contents are heated, or filtered, to kill, or remove, the secondary growth of resistant bacteria, and titrated for lytic activity by the serial dilution method,[3,4] one finds that the concentration of the lytic principle is at the same maximum in all the tubes in which lysis was observed

[5] Doerr, R., *Schweiz. med. Woch.*, 1923, iv, 1009.

[6] Doerr, R., and Rose, G., *Schweiz. med. Woch.*, 1924, v, 10.

[7] Reichert, F., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 235.

[8] Gildemeister, E., and Herzberg, K., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 228.

[9] Bronfenbrenner, J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 315.

earlier, irrespective of the amount of lytic filtrate they received orig-
inally. On the other hand, all the tubes receiving less than 10^{-10}
cc. of the filtrate show no lytic activity.

The results of the experiment illustrated in Protocol 1 suggest that
the lytic agent is present in the filtrate not in the form of true solution,
but in a particulate state. Thus, the fact that 10^{-10} cc. of the filtrate
produces lysis, whereas 10^{-11} cc. produces no lysis is explained as show-
ing that 10^{-10} cc. contains at least one and less than ten indivisible
active particles of bacteriophage. Since the distribution of such dis-
crete particles in very high dilutions may be such that not every cc.
of dilution will contain a particle, it is scarcely surprising that in the
next dilution (1×10^{-11} cc.) we find two tubes only (Nos. 2 and 8)
showing lytic activity.

If, then, it is true that 1×10^{-10} cc. contains one indivisible par-
ticle of the active principle, it should follow that one-half or one-third
of this amount will contain less than one, and hence show no lytic
action. If, however, the lytic agent is present in the form of a true
solution, combination of three portions of $1/3 \times 10^{-10}$ cc. should result
in the same activity as is produced by one whole portion of $1 \times
10^{-10}$ cc. Or, the lytic agent may be present in a measurable amount
in even 1×10^{-11} cc., which concentration, however, would appear too
low to induce a change in bacteria resulting in their lysis. In this
event a combination of 10 portions of 1×10^{-11} cc. should be active.
Should this prove to be the case, the true solubility, as opposed
to particulate distribution of bacteriophage, would be established.
The following experiment is one of a series of similar ones carried out
to obtain information on this point.

Active filtrate Laudman Shiga was diluted with sterile broth so that each cc. of
dilution contained 1×10^{-9} cc. of the original filtrate. 2 cc. of this dilution was
added to 198 cc. of sterile broth, mixed thoroughly, and distributed in 10 cc.
amounts in each of twenty sterile tubes. Thus each of the tubes of the series
received 1×10^{-10} cc. of the original filtrate (Protocol 2, A). In a similar manner
another series of twenty tubes was prepared to contain in each 1×10^{-11} cc. of the
original filtrate (Protocol 2, B). Each of the tubes was then seeded with 0.1 cc.
of a suspension of *Bacillus dysenteriæ* Shiga containing 10^9 susceptible bacteria per
cc. and incubated at 37°C. The appearance of lysis was recorded at the end of
18 hours, as well as at the end of 40 hours of incubation. At this time all the tubes
were heated to destroy the bacteria, and a drop of the contents was deposited on a

Protocol 1.*

Increase of Lytic Principle to a Maximum.

Amount of active filtrate in 0.1 cc. volume, cc.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}
Broth, cc.	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.9
Bacterial suspension, cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Incubated at 37° C. for 18 to 24 hrs.

Results	+	+	+	+	+	+	±	±	±	±	−	−	−

Incubated again for 18 hrs. at 37°C., heated for 30 min. at 56°C. (or filtered), and titrated.

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13
1 × 10^{-1} cc.	+	+	+	+	+	+	+	+	+	+	−	−	−
1 × 10^{-3} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1 × 10^{-5} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1 × 10^{-7} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1 × 10^{-9} "	+	+	+	+	+	+	+	+	+	−	−	−	−
1 × 10^{-10} "	−	+	+	+	+	+	+	+	+	−	−	−	−
1 × 10^{-11} "	−	−	−	−	−	−	+	+	−	−	−	−	−
1 × 10^{-12} "	−	−	−	−	−	−	+	−	−	−	−	−	−

* In this as well as in following protocols the sign + signifies clearing of culture (or lysis of bacteria); the sign ± signifies a partial clearing; and the sign − signifies absence of lysis.

surface of agar, previously seeded with susceptible bacteria (agar transfer). This procedure served as a final check on the lytic activity of the contents of each tube.

Protocol 2.

Particulate Distribution of the Lytic Agent.

Series A.

2×10^{-9} cc. of lytic filtrate in 2 cc. volume + 198 cc. of sterile broth. Distributed in a series of twenty tubes, each receiving 1×10^{-10} cc. of filtrate in 10 cc. volume.

	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.
Tube No......	1	2	3	4	5	6	7	8	9	10
Lysis.........	±++	±++	±++	±++	---	±++	±++	±++	---	±++
Tube No......	11	12	13	14	15	16	17	18	19	20
Lysis.........	---	±++	±++	±++	±++	±++	±++	±++	---	±++

Contents of Tubes 5, 9, 11, and 19 were now combined, filtered, and the filtrate was seeded with susceptible bacteria. No lysis was observed.

Series B.

2×10^{-10}cc. of lytic filtrate in 2 cc. volume + 198 cc. of sterile broth. Distributed in a series of twenty tubes, each receiving 1×10^{-11} cc. of filtrate in 10 cc. volume.

	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.
Tube No......	1	2	3	4	5	6	7	8	9	10
Lysis.........	---	---	---	---	---	---	---	---	±++	---
Tube No......	11	12	13	14	15	16	17	18	19	20
Lysis.........	---	---	---	---	---	---	---	---	---	---

Contents of Tubes 1 to 8 and 10 to 20 were all combined, filtered, and the filtrate was seeded with susceptible bacteria, but no lysis could be observed.

As will be observed from the results presented in Protocol 2, a great majority of the tubes of Series A, each containing 10^{-10} cc. of the original filtrate, showed lysis, as did also a few of the tubes in Series B.

Protocol 3.

Effect of the Concentration of Agar on Lytic Activity.

5 per cent beef infusion agar, cc.	9	9	5	5							
2 per cent beef infusion agar, cc.					5	5	2.5	2.5	2.5	1.5	1.5
Beef infusion broth, cc.	0.0	0.0	4	4	4	4	6.5	6.5	6.5	7.5	7.5
B. dysenteriae Shiga suspension, cc.	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
0.1 cc. Duggan Shiga bacteriophage diluted in H_2O.	$1:10^4$	$1:10^6$	$1:10^6$	$1:10^8$	$1:10^7$	$1:10^8$	$1:10^7$	$1:10^7$	$1:10^9$	$1:10^8$	$1:10^9$
Resulting agar concentration, *per cent.*	4.5	4.5	2.5	2.5	1.0	1.0	0.5	0.5	0.5	0.3	0.3

Incubated at 37°C. overnight.

Plaque count.	0	0	15	0	8	0	39	5	0	75	0
Maximum diameter of plaques, *mm.*	—	—	0.25	—	1	—	2.5	—	—	—	—
No. of plaques per cc. (titer).			0.00015×10^{10} $(\pm 15 \times 10^5)$		$\pm 0.1 \times 10^{-10}$		2.5 $\pm 0.5 \times 10^{10}$			4 $\pm 0.7 \times 10^{10}$	
No. of units per cc. as determined by broth titration.											1×10^{10}

The contents of the tubes showing lysis were discarded and those showing absence of lytic activity were now combined, filtered, seeded with susceptible bacteria, and incubated at 37°C.

Since combination of the contents of several tubes containing insufficient amount of lytic principle in each does not result in bringing about lysis, it appears that the lytic principle is distributed through the medium in the state of indivisible units.

Effect of Concentration of Agar on Lytic Titer.

The results of the preceding experiment indicate that the lytic agent is distributed in the medium as if particulate. If these particles are living and multiplying individuals subject to numerical evaluation, as suggested by the early work, their count by dilution in broth should correspond with that obtained on agar. However, as stated above, this is not the case.

While investigating to explain this discrepancy, it occurred to us that the ease with which lytic principle is known to be adsorbed on a variety of colloidal substances [10-12] may be responsible for the lowering of lytic activity when tested on agar. It seemed logical to suppose that by decreasing the agar content of the medium one might cause the plate counts to approach more nearly the values obtained by parallel broth dilution titrations. Accordingly, experiments were performed in which anti-Shiga as well as anti-*coli* lytic filtrates were plated out on agar, varying in concentration from about 4.5 per cent to 0.25 per cent. In parallel titrations the lytic activity of these filtrates was also determined by the broth dilution method. Protocol 3 outlines one of many of these experiments.

The lytic filtrate Duggan Shiga used in this experiment when tested in broth caused lysis in the amount of 1×10^{-10} cc. Thus, as seen from Protocol 3, when the concentration of agar is reduced below 0.5 per cent, the count of plaques per cc. obtained thereon is 7×10^9 or practically that obtained by the broth dilution method (1×10^{10}). On the other hand, when the concentration of agar is raised to 1 per

[10] Doerr, R., *Klin. Woch.*, 1922, i, 1489.

[11] Nakamura, O., *Arch. Hyg.*, 1923-24, xcii, 61.

[12] Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 422.

cent, the readings are only 10 per cent of those obtained by broth titration, and if the concentration of agar is further increased, the titer becomes progressively lower.

Effect of Agar Concentration on the Plaque Size.

In addition to the changes in the number of plaques observed as the concentration of agar was varied, we found that in general the average size of plaques tended to increase as the concentration of agar

Protocol 4.

Effect of the Concentration of Agar on the Activity of Purified Lytic Principle.

Agar concentration, per cent......	4.5	2.5	1	0.5
Amount of agar, cc..............	9	9	9	9 .
18 hr. culture of B. dysenteriæ Shiga, cc.....................	0.9	0.9	0.9	0.9
0.1 cc. of Duggan Shiga filtrate diluted.....................	$1:10^1$	$1:10^3$	$1:10^6$	$1:10^{10}$
Plaque count...................	0.0	130	180	281
No. of plaques per cc. (titer)	0.0	(130×10^4) 0.00013×10^{10}	(180×10^6) 0.018×10^{10}	(281×10^7) 0.28×10^{10}
Maximum diameter of plaques, mm........................	—	0.25	1	2.5

Plates from which the above figures were taken are shown in the photograph in slightly less than actual size (Figs. 1 to 4).

was reduced (Protocol 3). This ·phenomenon seemed to us of great interest since it suggests that the size of the plaques may be not only a function of a given lytic agent, as is usually believed, but it may also depend on the concentration of agar. Since the lytic agents used by us in these experiments were recently isolated by us from fecal material, they may conceivably have consisted of mixtures of different lytic agents which could account for the lack of constancy in the size of plaques.[13-18] We therefore purified these filtrates by repeated

[13] Gratia, A., Compt. rend. Soc. biol., 1923, lxxxix, 821, 824.

[14] Bail, O., and Watanabe, T., Wien. klin. Woch., 1922, xxxv, 169.

[15] Bruynoghe, R., and Appelmans, R., Compt. rend. Soc. biol., 1922, lxxxvii, 96.

[16] Wolff, L. K., and Janzen, J. W., Ann. Inst. Pasteur, 1923, xxxvii, 1064.

[17] Wagemans, J., Arch. internat. pharmacod. et thérap., 1923–24, xxviii, 159, 181.

[18] Matsumoto, T., Wien. klin. Woch., 1923, xxxvi, 759.

passages on agar, each time from a single plaque; when the lytic agents thus obtained appeared to give plaques of reasonable uniformity, we replated them on agar of varying concentrations (Protocol 4).

Similar changes in the size and number of plaques under the influence of variations in concentration of agar were observed with purified lytic filtrates (Phage B. W. and Pet. 2 respectively) sent to us by Gratia.[18]

When Irish moss was substituted for agar, results similar to those shown above were obtained. As the concentration of jelly was reduced the size and the number of plaques were increased.

Protocol 5.

Effect of an Excess of Susceptible Bacteria on the Size of Plaques.

Molten 2 per cent agar, cc.............	9	9	9	9	9	9
B. *dysenteriæ* Shiga 18 hr. culture, No. of bacteria in 1 cc..................	10^{10}	10^9	5×10^8	1×10^8	1×10^7	1×10^6
0.1 cc. Laudman Shiga filtrate diluted..	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$

Plates poured and incubated at 37°C. overnight.					
Maximum size of plaques, *mm*.........	0.25	0.5	0.5	0.75	Unsatisfactory discontinuous growth.

Effect of the Number of Susceptible Bacteria on the Number and Size of Plaques.

Since it is known that bacteriophage principle does not multiply except in the presence of bacteria, and, moreover, since it is generally assumed that the development of plaques is the result of lysis of bacteria surrounding or developing on the spot in close proximity with the particles of active agent, it is possible that the number and size of such plaques will also depend on the density of bacterial suspension subjected to lysis. This relation was studied by pouring a series of agar plates in which, all other factors being constant, the total number of susceptible bacteria was varied.

As can be seen from the results presented in Protocol 5, when the number of bacteria added was less than 100 million per cc., the growth was discontinuous and the plaques were not clearly demarcated. As

the number of bacteria increased, the size of the plaques diminished, the total count of plaques not being appreciably affected.

Effect of Relative Concentration of Young and Old Bacteria.

It is known that the lytic agent increases in titer only in the presence and at the expense of young susceptible bacteria; moreover, older bacteria are known to adsorb lytic agent without undergoing lysis, thus decreasing the concentration of free lytic agent in solution.[19-21]

Protocol 6.

Effect of Relative Number of Old and Young Susceptible Bacteria on Number and Size of Plaques.

Molten 1 per cent agar, cc....................	9	9	9	9	9	9	9
18 hr. culture of B. dysenteriæ Shiga, cc....	1.0	0.75	0.5	0.25	0.0	1	0.0
12 day culture of B. dysenteriæ Shiga, cc....	0.0	0.25	0.5	0.75	1.0	0.0	1
0.1 cc. of Laudman Shiga filtrate diluted.	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	0.0	0.0

Plates poured and incubated at 37°C. overnight.

Maximum size of plaques, mm..........	1	1	0.8	0.6	Discontinuous growth.	Even growth.	Discontinuous growth.
No. of plaques.........	240	105	82	20	Counting impossible.		

For these reasons it was thought that the relative concentration of young and old bacteria might also appreciably affect the appearance of plaques.

In order to investigate this question, emulsions of equal turbidity were prepared by suspending in physiological salt solution agar cultures of *Bacillus dysenteriæ* Shiga of 18 hours and of 12 days respectively. Varying amounts of each were added to the mixture of agar and lytic agents as indicated in Protocol 6.

[19] Adsorption experiment will appear in *The Journal of Experimental Medicine.*

[20] Jaumain, D., and Meuleman, M., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 362.

[21] Prausnitz, C., and Firle, E., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, suppl., 148.

This experiment shows that both the number and the size of plaques diminish as the proportion of old bacteria is increased. When old bacteria only were used, the growth was discontinuous and the plaques could not be distinguished.

Effect of Presence of Resistant Bacteria on the Appearance of Plaques.

Since, in the preceding experiment, it was found that the presence of old bacteria affects the appearance of plaques, it was thought useful to inquire into the effect of young resistant bacteria. For this pur-

Protocol 7.

Effect of Relative Number of Susceptible and Resistant Bacteria on the Number and Character of Plaques.

Molten agar 1 per cent, cc....	9	9	9	9	9	9	9
B. dysenteriæ Shiga susceptible, cc....	1.0	0.75	0.5	0.25	0.0	1.0	0.0
B. dysenteriæ Shiga resistant, cc....	0.0	0.25	0.5	0.75	1.0	0.0	1.0
0.1 cc. Laudman Shiga filtrate diluted....	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	0.0	0.0

Plates poured and incubated at 37°C. overnight.

Maximum size of plaques, mm....	1	1	1	1	—	Even layer of growth.	
Plaque count....	70	100	70	73	No		
Character of plaques....	Clear.	Fairly clear.	Faint.	Faint.	plaques.		

pose suspensions of susceptible and of resistant bacteria were prepared from respective 18 hour cultures on agar, and an experiment identical in other details with that above was performed.

The presence of resistant bacteria had no effect on either the count or size of the plaques (Protocol 7). However, the plaques appeared to the naked eye less distinct, were faint, as the proportion of resistant bacteria was increased. The pale plaques were altogether indistinguishable under the low power of the microscope from the background of bacterial growth.

Effect of Specificity of Bacterial Substratum on the Size of Plaques.

In dealing with several lytic filtrates of different origin we have observed several instances in which a given filtrate exhibiting activity against two or more species of bacteria produced plaques of different size, depending on the bacterium used. In order to see whether it is the bacterial substratum or the lytic filtrate which is the decisive factor determining the size of plaques, the following experiment was performed.

Laudman Shiga filtrate, which was also active to a less extent on *Bacillus coli*, was increased in its potency for the latter organism by a

Protocol 8.

The Size of Plaques Produced by Certain Lytic Principles on Cultures of Heterologous Bacteria.

	Laudman Shiga filtrate.		Coli filtrate.	
	Produced from cultures of Shiga bacillus.	Produced from cultures of colon bacillus.	Produced from cultures of colon bacillus.	Produced from cultures of Shiga bacillus.
Plated with................................	*B. dysenteriæ* Shiga. | *B. coli.*	*B. dysenteriæ* Shiga. | *B. coli.*	*B. dysenteriæ* Shiga. | *B. coli.*	*B. dysenteriæ* Shiga. | *B. coli.*
Maximum size of plaques, *mm*........	1.2 | 0.6	1.2 | 0.6	0.6 | 0.6	0.6 | 0.6

series of passages in broth until its titer for colon bacillus reached 10^9 units per cc. Likewise a lytic agent, originally lytic for colon bacillus, was transferred in a series of passages with Shiga bacillus. A sufficient number of transfers was made in each case to eliminate by dilution all traces of the original lytic filtrate. The original Laudman Shiga lysin was then plated in 1 per cent agar with both *Bacillus dysenteriæ* Shiga and *Bacillus coli*. The filtrate resulting from "adaptation" of this lysin to *Bacillus coli* was also plated with both organisms. Similarly, the lytic agent originally active upon colon bacillus, and the product of its "adaptation" to Shiga bacillus, were each plated with both organisms as shown in Protocol 8. The concentration of agar was in all cases 1 per cent.

It appears from the above experiment that the Laudman lytic agent, whether produced in cultures of *Bacillus dysenteriæ* Shiga or in those of *Bacillus coli* retained the characteristic of forming larger plaques with *Bacillus dysenteriæ* Shiga and smaller plaques on plates of *Bacillus coli*. The lytic agent anti-*Bacillus coli* produced a plaque of about 0.6 mm. in diameter when grown with either *Bacillus dysenteriæ* Shiga or *Bacillus coli* irrespective of the culture from which the filtrate was obtained.

The size of the plaque formed on plates of a given bacterium is, then, a function of the lysin, and is retained without change through the process of so called adaptation.

<div align="center">DISCUSSION.</div>

The results of our experiments indicate that the lytic principle acts as though it were present in the medium in the form of indivisible units. While it was suggested by d'Hérelle that these active units are actually organized living particles, the evidence so far produced by him in support of this view does not seem convincing. Since active filtrates consisting of water with a very small percentage of solids, comprising, in addition to active principle, salts, tissue extractives, metabolic products, and bacterial debris, can still be diluted 10^{10} times before reaching the limit of their activity, it is equally possible to suppose that the units of active agent are molecules of some inanimate material.

It is also possible that lytic substance is present in the medium in a state of true solution, and is merely adsorbed on bacterial debris and, being distributed with it, appears as being particulate itself. In such case the number of particles of bacterial debris present will determine indirectly the particulate concentration of active agent. This possibility is especially suggestive if it is remembered that the highest number of active units present in a lytic filtrate at the height of its activity is about 1×10^{10} per cc.—a concentration which very closely approaches the figure expressing the peak of the concentration of susceptible bacteria in a cc. of the medium.

Furthermore, the particles of lytic agent do not behave as if they were living organisms, as shown by the effect on them of comparatively slight changes in concentration of agar. Even as slight an increase

in consistency of the agar as that from 1 per cent to 2.5 per cent suppresses over 99 per cent of plaques. Moreover, it is difficult to reconcile the changes in the size of plaques, as brought about by various procedures in our experiments, with the view of d'Hérelle that the plaques represent the "colonies" of bacteriophage. For instance, if the size of the plaque depends on the progress of "invasion" of bacteria by the bacteriophage why should it be affected by the concentration of agar? Moreover, the more numerous the susceptible bacteria around the initial particle, the more they should be "invaded;" each of these, according to d'Hérelle, in turn should become a source from which the next generation of bacteriophage would invade the nearest bacteria, and in consequence the larger should be the plaque, or at least it should not be smaller. As a matter of fact, however, the greater the concentration of susceptible bacteria, the smaller are the plaques. Similarly it would be difficult to explain, on the basis of d'Hérelle's hypothesis, why both the number and the size of plaques decrease with the increase in proportion of *old* bacteria which are not subject to lysis, whereas the increase in concentration of young *resistant* bacteria leaves both the number and the size of plaques unaffected.

On the other hand, a logical explanation of these phenomena can be given on the basis of the observed tendency of agar and of old bacteria to adsorb the lytic agent, thus interfering with the progress of its diffusion. Since resistant bacteria do not adsorb the lytic principle, other conditions being equal, the presence of an excess of resistant bacteria does not interpose an obstacle for normal diffusion of the lytic agent—hence the difference in their respective effect on the size of the plaques.

SUMMARY AND CONCLUSIONS.

The experiments reported above confirm the fact that lytic principle is distributed in active solution in a state of indivisible units. This permits its quantitative evaluation by serial dilution, as well as by plating on agar. The latter method, however, often gives readings considerably lower than those obtained by the broth dilution method of titration. By varying the concentration of agar it has been possible to show that the discrepancy is due to adsorption of the lytic agent on agar. When the concentration of the latter is increased from 0.3 per

cent to 2.5 per cent the number of plaques of lysis is reduced more than 100 times. At the same time the average size of the plaques also decreases approximately to one-tenth of the original.

The size, as well as the number of plaques, has been found to depend also on the condition of the culture employed in titration. Thus, when the culture exposed to the action of lytic agent is composed of young susceptible bacteria, the greater the concentration of bacteria, the smaller the plaques.

When the culture is composed partly of young and partly of old susceptible bacteria, both the size and the number of the plaques are diminished with the increase in the relative concentration of old bacteria. On the other hand, presence in the culture of resistant bacteria does not affect either the size or the number of the plaques so long as the relative concentration of susceptible bacteria in the culture is sufficient to allow formation of them. The plaques appearing in the presence of a high concentration of resistant variants in the culture are relatively indistinct owing to overgrowth.

Under carefully controlled conditions the size of plaques is found to be determined by the character of the lytic filtrate. Thus in the case of lytic agents which act upon more than one bacterial species the size of the plaques remains constant, irrespective of the bacterial substratum used for the production of the active filtrate.

<div align="center">EXPLANATION OF PLATE 20.</div>

FIG. 1. 4.5 per cent agar $+ 0.1 \times 10^{-1}$ cc. or $100,000 \times 10^{-7}$ cc. of phage—no plaques.

FIG. 2. 2.5 per cent agar $+ 0.1 \times 10^{-4}$ cc. or 100×10^{-7} cc. of phage—130 plaques 0.25 mm. in diameter.

FIG. 3. 1 per cent agar $+ 0.1 \times 10^{-7}$ cc. of phage—180 plaques 1.0 mm. in diameter.

FIG. 4. 0.5 per cent agar $+ 0.1 \times 10^{-7}$ cc. of phage—281 plaques 2.5 mm. in diameter.
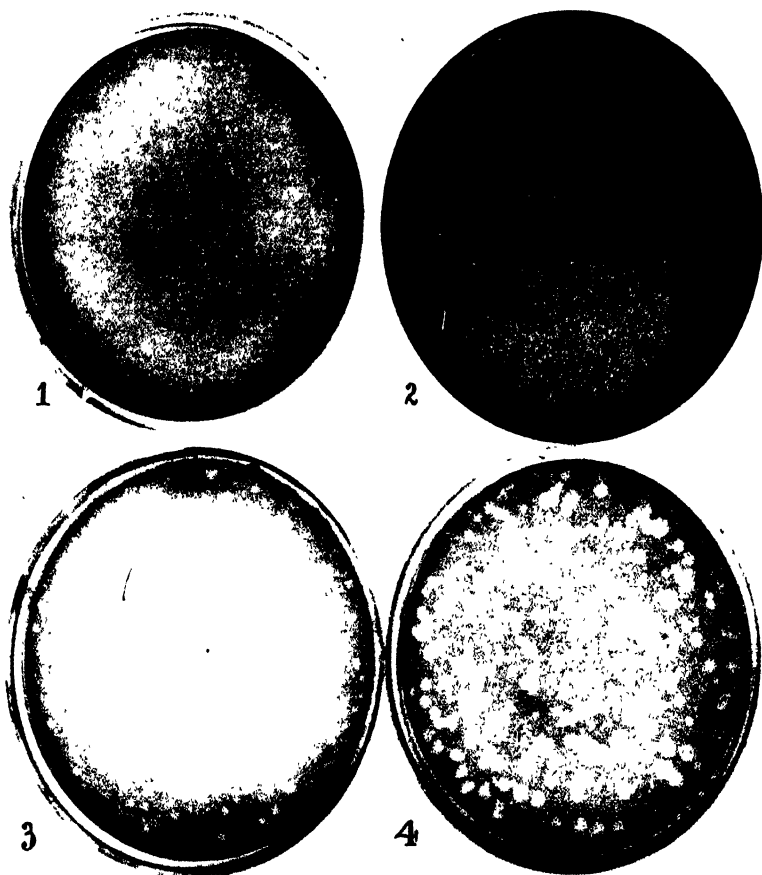
STUDIES ON UROBILIN PHYSIOLOGY AND PATHOLOGY.

V. The Relation between Urobilin and Conditions Involving Increased Red Cell Destruction.

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Much of the interest in urobilin has come from the fact that it is found in the urine of patients suffering from diseases characterized by an excessive destruction of red blood cells. The occurrence of urobilinuria has been reported in hemolytic icterus, pernicious anemia, malaria, and under various circumstances involving hemorrhage, as, for example, those of hemophilia, apoplexy, and hemothorax.[1-3] There have been many clinical studies in such connection, but little experimental work has been reported. and despite a general belief in the importance of the phenomenon, no uniform conception has developed of its pathological or diagnostic significance in the conditions mentioned.

Many workers have assumed that urobilin may be manufactured directly from the hemoglobin or bilirubin accumulation in the tissues as result of blood extravasation and destruction. The pigment thus produced is supposed to be carried by the blood to the kidneys and excreted by the urine. And yet Kunkel[4] was unable to show this experimentally; and Quincke[5] repeatedly attempted to demonstrate urobilin in old blood clots without success.

The presence of large amounts of urobilin in the stools of patients suffering from hemolysis has long been recognized. Indeed, the estimation of the pigment in the feces has, in recent years, been taken as an approximate measure of the amount of red cell destruction.[6] Yet, many extraneous factors have been shown

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[1] Hoppe-Seyler, G., *Virchows Arch. path. Anat.* 1891, cxxiv, 30.

[2] Meyer-Betz, F., *Ergebn. inn. Med. u. Kinderheilk.*, 1913, xii, 733.

[3] Eppinger, H., Die hepato-lienalen Erkrankungen, p. 76, in Enzyklopædie der klinischen Medizin, Berlin, 1920.

[4] Kunkel, A., *Virchows Arch. path. Anat.*, 1880, lxxix, 455.

[5] Quincke, H., *Virchows Arch. path. Anat.*, 1884, xcv, 125.

[6] Robertson, O. H., *Arch. Int. Med.*, 1915, xv, 1072.

to influence in practice the output of fecal urobilin. Even in healthy animals these cause wide fluctuation in the values.[7]

In our earlier papers the normal physiology of urobilin has been studied.[7-9] The communication[10] immediately preceding the present one dealt with conditions of liver damage and biliary obstruction. We are here concerned with the effects of increased blood destruction and allied conditions. For the purposes of the investigation the quantitative measurement of urobilin was made by a method which has already been described.[8] The errors encountered in the study of bile obtained from the open fistula of Schwann have been avoided by a sterile method of intubation,[7, 11] which renders possible precise determinations of the entire liver output

Methods.

The general methods adopted for the intubation of the bile ducts of dogs, care of the animals, and the daily collection of and pigment determinations on urine, stool, and bile have already been described.[7,8] All the animals were permanently intubated, some for the collection of the total bile, others for that of but a sample of the liver output, while still others were so intubated as to permit the bile to flow either into the duodenum or to the collecting bag at the will of the observer. Every day or two portions of the collected bile were incubated with agar to determine whether it had become infected, and furthermore, centrifugalized specimens were frequently examined to the same end. When bacteria were found the animal was discarded for the purposes of this work. All the experimental procedures were carried out under aseptic conditions.

In some instances the effects of an excessive destruction of red blood cells, consequent on intercurrent disturbances, were studied, while in others destruction was induced by the giving of a hemolytic substance or by the intravenous injection of distilled water. Several instances of localized infection, especially of the respiratory tract, accompanied by increased blood destruction, came under observation.

Toluylenediamine in aqueous solution causes a marked red cell destruction.[12] It was given, through a stomach tube, in doses averaging 0.05 to 0.1 gm. per kilo of animal. Icterus developed, yet the dogs remained active and in what appeared

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[7] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.

[8] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xli, 503.

[9] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 719.

[10] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xlii, 99.

[11] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

[12] Eppinger, H., Die hepato-lienalen Erkrankungen, p. 124, Berlin, 1920.

to be good condition. Toluylenediamine not only damages the red cells, however, but causes more or less severe liver injury.[10] Sodium oleate, 50 to 75 cc. of a 1 per cent aqueous solution, was given to some animals intravenously on successive days.

To produce an immediate hemolysis distilled water was employed. It was either given directly into a vein,—in amounts of 250 to 300 cc. of it to animals of about 10 kilos during a period of 3 to 5 minutes,—or 100 to 150 cc. of blood was withdrawn, shaken with 200 to 250 cc. of sterile water to hemolyze it, and the mixture reinjected after straining it through a cotton mesh.

The effects of the extravasation of blood into the tissues could be studied as an incidental result of the intubation, since the operative procedures inevitably involved more or less hemorrhage into the tissues about the incision. In certain cases artificial hematomas were produced by the subcutaneous injection of sterile blood, usually obtained from the animal's own vein.

For special purposes, large amounts of urobilin-free bile (two to three times the ordinary 24 hour output of the animal) were given by gavage. To imitate the increased passage of bilirubin into the intestine which follows upon increased blood destruction this pigment was given by gavage on one occasion. It had been prepared from fresh dog bile by a method already described.[9]

In all of the cases the percentage of the circulating hemoglobin was determined daily by the Newcomer method, following a procedure already described.[7] This percentage varies somewhat from day to day and in the absence of any complication the output of bilirubin in the bile of intubated dogs tends to vary in parallel with it.[13] The relation that exists between the two pigments is not strictly quantitative, yet it is sufficiently close to give ground for the supposition that excessive blood destruction is going on whenever any marked increase occurs in the bilirubin output.[14]

Absence of Urobilinuria during Increased Blood Destruction under Conditions Involving Total Bile Loss.

It may be recalled from our previous papers that when the common duct is so intubated that none of the bile reaches the intestine but the whole is lost to the organism, urobilin disappears from this secretion as also from the stools and urine;[7] and even serious damage to the liver does not cause it to reappear.[10] The same holds true when the damage has been to the blood, or to both blood and liver, as in the case of toluylenediamine.

[13] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 733.
[14] Rous, P., *Physiol. Rev.*, 1923, iii, 75.

Specimen Protocols.

I. Female dog, weight 9¾ kilos (see Text-fig. 1).

Under ether, intubation of the common duct was performed. By the 3rd day the stool and urine had become urobilin-free, and they remained so thereafter.
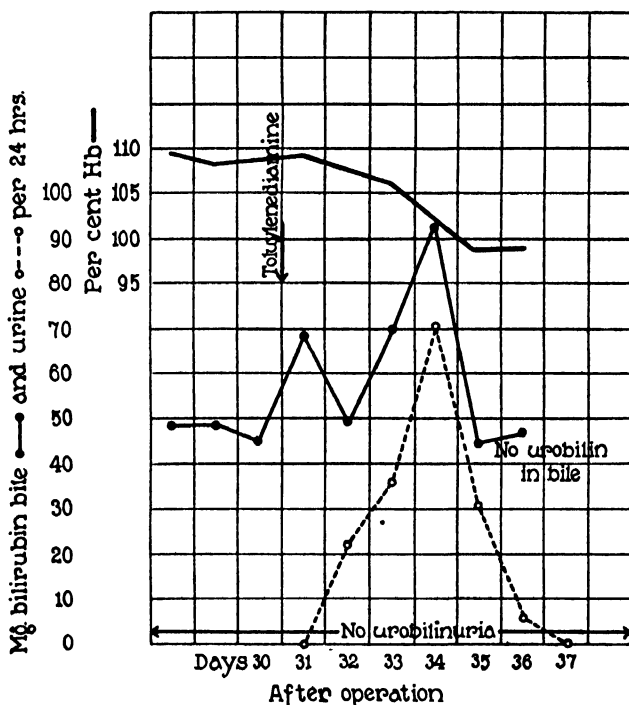
TEXT-FIG. 1. Absence of urobilinuria during pathological blood destruction in a dog losing the total bile.

Toluylenediamine in aqueous solution was given by mouth to an animal with intubated common duct. There resulted a progressive fall in the percentage of circulating hemoglobin, and the bilirubin output nearly doubled. There was a marked bilirubinuria and tissue icterus, yet no urobilin was to be found in the bile or urine, and the stools remained consistently acholic and practically free from urobilin (see Protocol I).

1 month after operation the animal was healthy and active; and the bilirubin output of the bile was constant at about 50 mg. in 24 hours. A small dose (0.475 gm.) of toluylenediamine was now given. There resulted, as the chart shows, a progressive fall in hemoglobin; and a tissue icterus developed which persisted several days. The bilirubin output in the bile reached a maximum of 92 mg. in the fourth 24 hour specimen after administration of the hemolytic agent. Bili-

rubinuria was marked. At no time however was urobilin found in the bile or urine. The stools contained about 2 or 3 mg. in each 24 hours. The presence of this amount is to be attributed to the escape of bile pigment from the jaundiced wall of the gut into the lumen, with subsequent bacterial action.[7]

II. Male dog, weight 10¼ kilos (see Text-fig. 2).

The common duct had been cannulated under ether anesthesia, and daily collections of the bile made. It remained sterile, and, after the 3rd day, contained no urobilin.
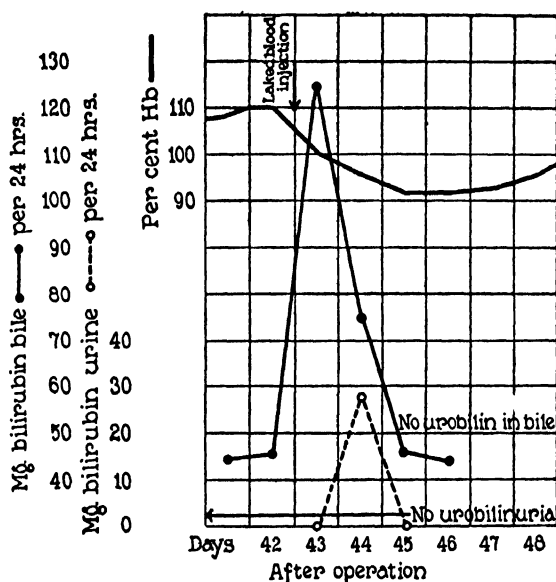
TEXT-FIG. 2. Absence of urobilinuria in a dog losing the total bile and injected with hemolyzed blood.

A portion of the animal's own blood laked outside of the body with distilled water was injected intravenously. The drop in hemoglobin was 18 per cent, and there was hemoglobinuria and bilirubinuria, yet no urobilin was found at any time in the urine, bile, or feces (see Protocol II).

After 6 weeks collection of bile specimens, 100 cc. of blood was withdrawn from the jugular vein of the animal, shaken with 200 cc. of sterile, distilled water, and the whole reinjected intravenously after straining. The injection required 3 minutes. The bilirubin output rose in the next 24 hours from the "normal" of 45 mg. to 125 mg. The urine contained large amounts of hemoglobin, and, on the day following, much bilirubin. The hemoglobin percentage fell 18 points. In 3 days the bile and urine had returned to normal. At no time was urobilin found in the bile, the urine, or the stools.

Urobilinuria after Increased Blood Destruction under Conditions
of Partial Bile Loss.

In the course of our previous work it was shown[7] that when only a fraction of the bile is collected each day, with the rest flowing into the intestine as usual, urobilin continues to be a constituent of both bile and stools, while furthermore, upon liver injury,[10] urobilinuria develops. Sodium oleate exerts a direct hemolytic action on the red cells, even *in vitro*,[15] but there seems to be no evidence that it injures the liver. Following the giving of it urobilinuria is usually not pronounced. Toluylenediamine injures the liver as well as the blood,[12] and it might be expected from what has just been said, that a marked urobilinuria would follow the giving of it, as is the actual case. The amount of urobilin in the stools increases greatly, in corollary to the increase in the amount of bilirubin reaching the bowel as the result of blood destruction.

The difference in the degree of urobilinuria in the two instances selected for illustration may lie in the fact that toluylenediamine, unlike sodium oleate, causes liver damage, but the protracted action of the substance would in itself lead to a sustained and perhaps a cumulation effect. The effect of the intravenous injections of sodium oleate is practically immediate and may be completed within a few minutes. The fall in the hemoglobin percentage was about the same in each of the instances now to be given.

Specimen Protocol.

III. Male dog, weight 10¾ kilos (see Text-fig. 3).

30 days after a cannula had been placed in the left lateral bile duct under ether anesthesia (draining 27 per cent of the liver) the animal received 1.2 gm. of toluylenediamine. The findings for the first 3 days thereafter, when there was a transient, if almost complete, bile suppression, have been discussed previously in another connection.[10] Here we are concerned only with the occurrences after the resumption of the bile flow.

On the 4th day after giving the drug, or the 37th of intubation, the drop in the hemoglobin percentage had become well defined, and it reached its lowest point 3 days later. During this later period of the progressive blood destruction there took place an enormous increase in the output of bilirubin and urobilin in the bile of each day. The polycholia found expression a little later in a great increase in

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[15] McPhedran, W. F., *J. Exp. Med.*, 1913, xviii, 527.

the urobilin of the stools. The disturbances subsided gradually, and in 10 days after the giving of the toluylenediamine its effect was almost spent.

The urobilinuria attendant upon the blood destruction was intense, and in general followed the curve representing the increase in bilirubin elimination in the
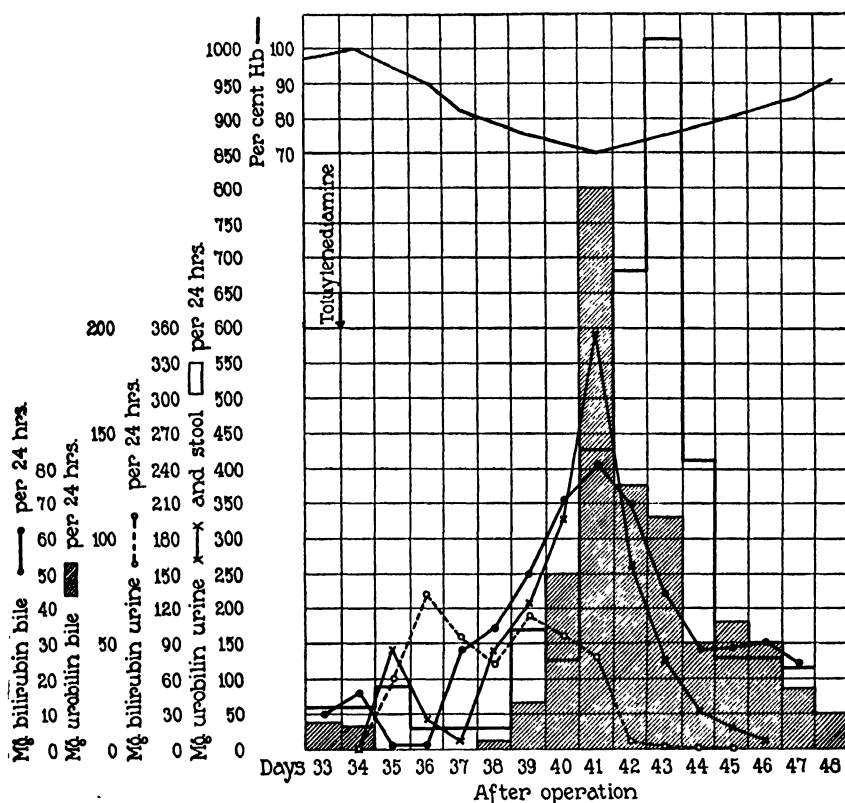
TEXT-FIG. 3. Urobilinuria during excessive blood destruction in a dog losing but a fraction of the total bile.

Toluylenediamine in aqueous solution was given by stomach tube. The findings for the first 3 days, when there was almost complete bile suppression, have been discussed before.[10] With the resumption of bile flow, and the excretion of bile pigment in great quantity, the bilirubinuria consequent upon the suppression lessened and disappeared, whereas urobilin appeared in stool and urine, the quantity soon becoming great and only lessening again as the output of bilirubin in the bile lessened. Concomitantly there was a parallel increase and then a decrease in the urobilin of the bile. These changes were attended by a fall in hemoglobin of 30 per cent—another gauge of the degree of blood destroyed (see Protocol III).

bile. Bilirubinuria was somewhat less pronounced and of shorter duration, as would follow from the circumstance that the bile flow was not long impeded. Thus bilirubinuria disappeared the day after the peak of the blood destruction was reached, while urobilinuria continued for 3 more days. Such a relationship, which will be further illustrated in experiments to be described, is characteristic of the two pigments in conditions characterized by excessive hemolysis.
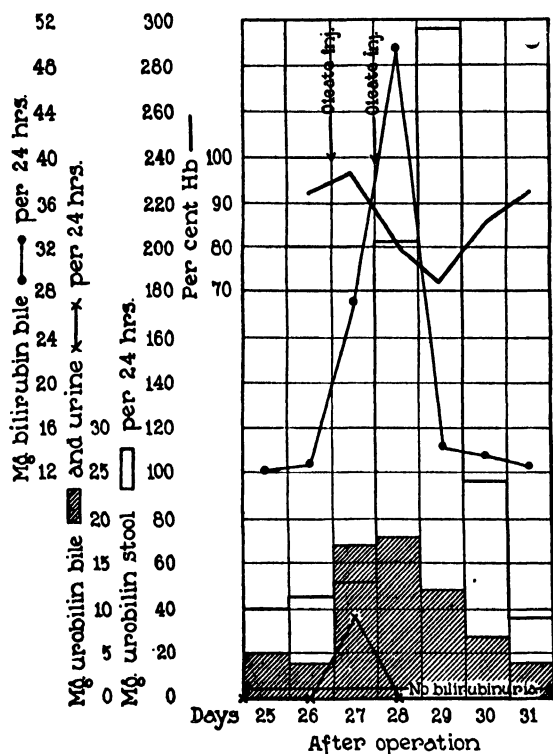
TEXT-FIG. 4. Urobilinuria during excessive blood destruction in a dog losing but a fraction of the total bile.

Intravenous injections of sodium oleate were given on successive days. The blood destruction was reflected in a tremendously increased output of bilirubin in the bile. There was a slight urobilinuria when the disturbance was at its height, but no bilirubin ever was found in the urine (see Protocol IV).

Specimen Protocol.

IV. Male dog, weight 12 kilos (see Text-fig. 4).

The intubation under ether anesthesia made it possible to collect each day the bile from the left lateral lobe of the liver, which was found later at necropsy

to comprise 32 per cent of the organ. The bile remained sterile and showed, like the stool, an approximately uniform content of urobilin.

4 weeks after the operation, 50 cc. of a 1 per cent solution of sodium oleate in water was injected intravenously on 2 successive days. There resulted a drop of 20 points in the hemoglobin percentage. The bile of the 24 hours after the first injection contained four times the usual amounts of bilirubin and urobilin. The stool also contained increased amounts of urobilin. There was a further increase in the pigments after the second injection, but 48 hours later the conditions had returned to the previous "normal." There was a transient urobilinuria but no bilirubinuria.

Urobilinuria during the Extravasation of Blood after Operation.

Immediately after the operation to intubate a common duct the output of bile pigment is greater than at later times, and it is especially prone to be so during the second or third 24 hours of bile collection.[13,16] It has been pointed out that this is, in part, to be accounted for by the pigment derived from blood extravasated during the operative manipulations. The possibility that the anesthetic causes hepatic injury must also be borne in mind in connection with the urobilin findings now to be described.

When the common duct has been intubated, with result that all of the biliary secretion is lost to the organism, the animal rapidly becomes urobilin-free, there being none of the pigment in bile, urine, or stools,[7] despite the blood extravasation and potential liver injury just referred to. But when, on the other hand, the bile from one portion of the liver only is collected while the rest flows as usual to the gut, there is almost regularly urobilinuria following the intubation. In only one instance out of eighteen has it been absent. It develops at a time when animals with intubated common duct are becoming urobilin-free.

The amount of the pigment often does not exceed 3 to 4 mg. per day during the 2 or 3 days that it is present. But in one instance it was abundant (Dog 4), an instance in which the abdominal incision had become infected and purulent. During 4 days, when the infection was at its height, 175 mg. of urobilin was excreted in the urine and the 24 hour bilirubin content of the bile rose from 70 mg. to 103,

[16] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

107, and 100 mg. on 3 successive days. Even then there was no bilirubinuria. Thereafter the purulence lessened rapidly and the incision healed.

Specimen Protocols.

V. Male dog, weight 12¼ kilos (see Text-fig. 5).

The urine for the 5 days preceding operation was urobilin-free. Intubation under ether anesthesia was performed in such wise that two-thirds of the biliary
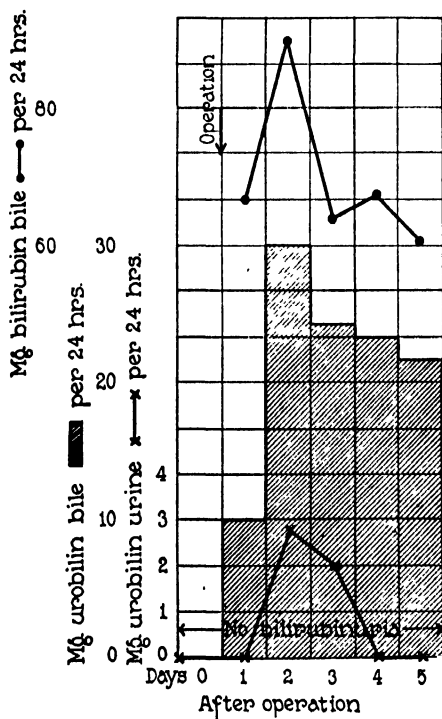
TEXT-FIG. 5. Postoperative urobilinuria in a dog losing but a fraction of the total bile.

There was the usual postoperative increase in the bilirubin and urobilin output in the bile. The urobilinuria of the 3rd and 4th days was slight. No bilirubinuria was noted at any time (see Protocol V).

secretion reached the duodenum through the normal channels, while the rest was collected. On the 2nd postoperative day the 24 hour bilirubin content of bile sample rose from 66 to 90 mg. and that of urobilin 10 to 30 mg. The urine during these 2 days showed a mild urobilinuria, and no bilirubin. On the 4th day conditions had returned to normal.

VI. Male dog, weight 12½ kilos (see Text-fig. 6).

Under ether the gall bladder was removed and an "altercursive" intubation was carried out,[7] two cannulas being inserted into the common duct which was cut between. The upper cannula and tube collected all the bile from the liver, while the lower one connected with the duodenum through the ampulla of Vater. The
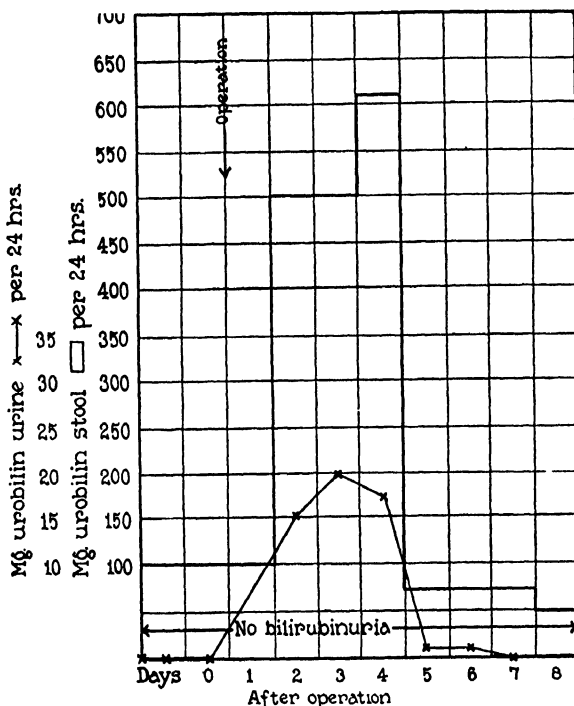
TEXT-FIG. 6. Urobilinuria followin operation in a dog with no bile loss.

An "altercursive" intubation was performed[7] and the total bile allowed to flow into the intestine after operation. The effect of the increased blood destruction, from the absorption of the extravasate due to trauma of the operation, was thus uninfluenced by any bile loss The urobilin findings resemble those in the preceding experiment, as in it bilirubinuria was absent (see Protocol VI).

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tubes were so arranged that all the bile issuing through the upper one was passed on through the lower into the duodenum, unless some was wished for examination, when it was diverted into a balloon. None was thus diverted during the period now under consideration.

The 24 hour urine specimens, urobilin-free for 3 days preceding the intubation, contained the pigment on the 2nd postoperative day, and for several days thereafter. No urine had been voided on the 1st day. The urobilinuria lasted

longer than usual.   No bilirubinuria was found at any time, indicating a complete ability of the liver to handle this pigment.   Since no bile was collected, our only measure of the postoperative polycholia was the stool urobilin which was, for a brief period, notably abundant.

Absence of Urobilinuria during the Absorption of Extravasated Blood from Hematomas.

The effects of extravasation of blood into the tissues many days after operation, when there was no suspicion of hepatic injury, were also studied.   In seven animals, three draining the total bile, and four, partial bile, artificial hematomas were produced, in the cervical region and in the abdominal walls, by subcutaneous injections of sterile blood, 30 to 60 cc., obtained from the animal's own vein.   In all the instances there followed an increase in the bilirubin output through the bile but no urobilinuria.

Urobilinuria Associated with Excessive Blood Destruction during Infections.

Many animals showed evidence of increased blood destruction during infections, as revealed by an increase in the bilirubin output in the bile.   These cases are therefore included in this report.   One such instance, of infection of a healing incision, has already been referred to, and other similar ones could be cited.   In such cases the relative parts played in the causation of the urobilinuria by the operation, the blood extravasation incident thereto, and the infection could not be discriminated.

The most common infection was distemper with which many dogs suffered, particularly during the early spring months.   In such of the distemper animals as were losing the total bile urobilin was never found, whereas in those losing but a fraction of it, or none at all ("altercursive" intubation) the affection frequently brought on urobilinuria.   The finding has special interest in view of the fact that urobilinuria is encountered during lobar pneumonia in human beings with intact bile ducts.   ·

Specimen Protocol.

VII. Female dog, weight 24 kilos (see Text-fig. 7).

6 days after the operation for intubation of one of the branches of the hepatic duct, under ether anesthesia, the animal developed a respiratory infection, be-

ginning with a purulent nasal discharge, and becoming gradually worse until there was frank dyspnea. It was somewhat prostrated, ate very little, and lost its former activity. The severe symptoms lasted only 3 days, and recovery was complete within a week.

On the 2nd day of the animal's illness the amount of bile elaborated by the intubated portion of the liver fell from 97 to 87 cc. but the secretion was darker and
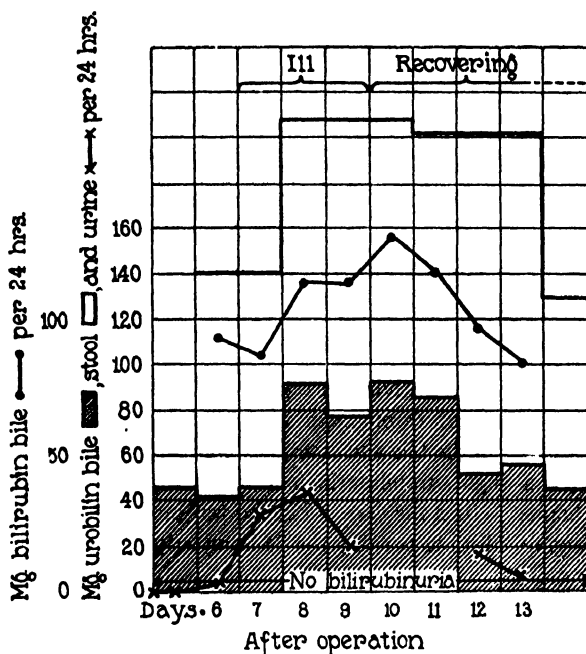
TEXT-FIG. 7. Urobilinuria consequent on respiratory infection in a dog losing but a fraction of the total bile.

The animal had a mild case of distemper characterized by a purulent nasal discharge, a conjunctivitis, and dyspnea. During the disease there was an increase in blood destruction, as shown by the increased output of bilirubin and urobilin in the bile. At the height of the infection there was a mild urobilinuria, but no bilirubinuria (urine specimens on days 10 and 11 were lost and hence are not represented on the chart). The animal recovered in a week (see Protocol VII).

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evidently more concentrated, and there was an absolute increase in the content of bilirubin and urobilin. The biliary changes continued for 3 days after symptoms had disappeared. Urobilinuria of mild degree appeared early, but it diminished and disappeared as the bilirubin output returned to the "normal." There was at no time any bilirubin in the urine. (The urobilinuria figures for days 10 and 11 are not shown on the chart since urine specimens were lost on these days.)

Urobilinuria after the Giving of Large Amounts of Bile by Gavage.

In a preceding paper of the present series[9] the fact has been recorded that when each day there is returned, through a stomach tube, to an animal losing all of the bile after intubation the amount of bile lost, urobilin reappears in the bile and stool, only to disappear again soon after the feedings are stopped. No urobilin appears in the urine as long as the amount of bilirubin given does not exceed the normal pigment output of the liver.

Through inadvertence a dog was one day given a specimen of urobilin-free bile containing two and one-half times the usual amount of bilirubin. To our surprise, the urine next day contained large amounts of urobilin, though none had been found before. The experiment was many times repeated on the same animal, and on others, always with the same result. The urobilinuria was less when the excess of pigment fed was lessened.

Example.—In Dog 25 the giving of 75 mg. of bilirubin per day,—approximately the amount it lost through an intubated common duct,—caused the appearance of urobilin in the bile and stool, but none in the urine. When 100 mg. was administered a slight urobilinuria followed,—5 mg. of the pigment being excreted in 24 hours. When the dose of bile fed was increased so that the dog was receiving 250 mg. of bilirubin per day the 24 hour output of urobilin in the urine was over 100 mg.

The same experiment was performed on dogs losing only a fraction of their liver output. With them, too, the giving of large amounts of bile by gavage was attended by the development of urobilinuria. In an instance selected for illustration (Protocol IX) the result was attained by feeding a solution of pure crystalline bilirubin prepared from dog bile.[9] We have not sought to learn precisely how considerable an excess of bile must be fed in order to produce urobilinuria. But certainly the excess need not be great. Under the circumstances of the feeding a great deal of bile passes along the intestine at one time—a very different state of affairs from the normal—and doubtless urobilin is formed and absorbed, *en masse*, so to speak, with result that some gets by the liver. In extreme instances, when very great amounts of bile are fed, there may be bilirubinuria as well, an occurrence which Schiff[17] long ago noted.

[17] Schiff, M., *Arch. ges. Physiol.*, 1870, iii, 598.

Specimen Protocols.

VIII.   Male dog, weight 16¼ kilos (see Text-fig. 8).

Under ether anesthesia, the common duct was intubated 20 days prior to the experiment.   The bile remained sterile, and it contained no urobilin after the 3rd
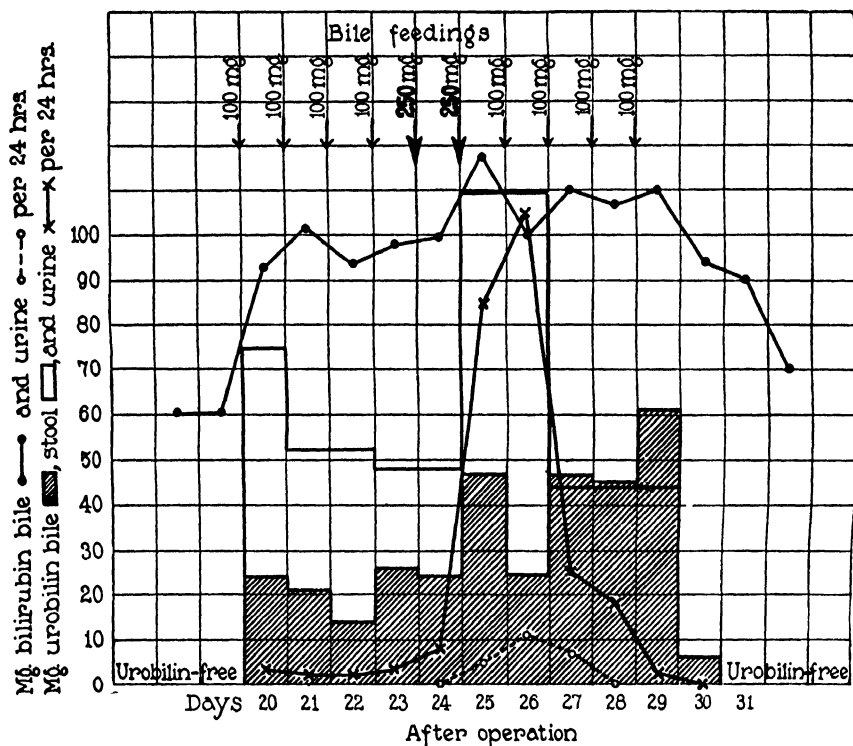
TEXT-FIG. 8.   Urobilinuria and slight bilirubinuria following large bile feedings to a dog losing the total bile.

Bilirubin was returned to the intestine in measured amounts by feeding bile through a stomach tube each day.   When the specimen contained 100 mg. of this pigment, urobilin reappeared in the bile and stools, previously free of it.   Increasing the amount to 250 mg. for 2 days precipitated a marked urobilinuria, though only traces were present before.   It decreased on the resumption of the usual feedings.   The stool urobilin showed a parallel rise.   Bilirubinuria was slight. 48 hours after feedings had ceased no urobilin was to be found (see Protocol VIII).

day.   On the 20th day specimens of fresh dog bile (liver bile) were fed by stomach tube each day for 10 days.   The content of bilirubin was uniform, 100 mg., except on two occasions, the fifth and sixth, when 250 mg. was given.   All the specimens fed were urobilin-free.

The first four feedings (each containing 100 mg. of bilirubin—considerably more than the 60 mg. lost) were attended by an increased output of this pigment by the liver, and the appearance of appreciable amounts of urobilin in bile and stool, and of a small quantity (3 to 4 mg. for 24 hours) in the urine.   Similar findings in many experiments of this kind have been described in a previous paper.[9] Following the giving of larger amounts of bile, containing over double the amount of bilirubin (250 mg.), the urobilinuria became a striking phenomenon, 205 mg. of the substance appearing in the urine during 72 hours, while there was a slight
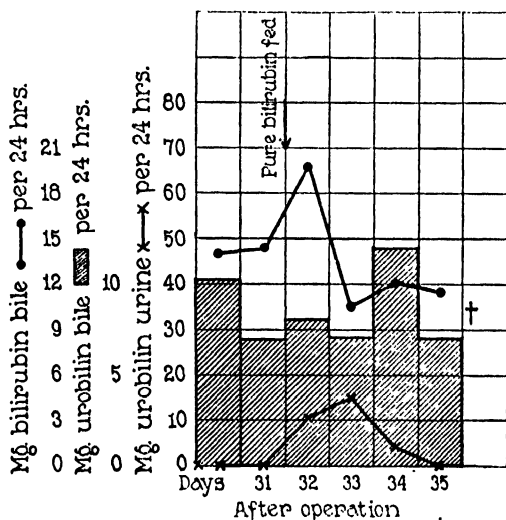
TEXT-FIG. 9. Urobilinuria following the feeding of pure bilirubin to a dog losing but a fraction of the total bile.

100 mg. of crystalline dog bilirubin in solution was fed by gavage,—about twice the amount lost by the fractional drainage.   The urine, previously urobilin-free, contained, in the next 72 hours, about 30 mg. of this pigment (see Protocol IX).

bilirubinuria as well.   The amount of urobilin fell off as less bile pigment was administered.   When the bilirubin fed each day as bile was reduced to 100 mg., the bilirubinuria disappeared, and when the bile feedings were discontinued the animal, within 48 hours, became again entirely urobilin-free, there being no longer any trace of this pigment in the stool or bile.

IX. Male dog, weight 12 kilos (see Text-fig. 9).

32 days after the operation to cannulate a branch of the hepatic duct, under ether anesthesia, at which time the urine was free from bilirubin and urobilin, and the pigment content of the bile sample was uniform from day to day, 100 mg. of pure crystalline bilirubin,—about twice the amount lost daily by the animal,— which had been prepared from fresh dog bile by a method already described,[9] was fed through a stomach tube.

The urine collected 18 hours later contained urobilin and continued to carry it for 48 hours more. Curiously enough, the bile showed no change in its content of this pigment, though it contained more bilirubin than previously. The disturbance was over in 3 days. 5 days after the feeding *B. subtilis* was found in the bile for the first time, doubtless as an air contaminant that had entered the tube system during the manipulations to empty the collecting balloon. The animal was chloroformed at once. No pathological changes were found at autopsy. The cannula was draining 32 per cent of the liver tissue.

<div align="center">DISCUSSION.</div>

The experiments should lead, it would seem, to a better understanding of the relation between urobilin and conditions characterized by excessive blood destruction.

In previous papers[7,10,16] we have proved that normally the formation of urobilin takes place only in the gastrointestinal tract. Even when the liver is seriously injured, or there is biliary obstruction with jaundice, no evidence is to be found of the participation of the tissue cells in general, or of the hepatic cells in particular, in the change of bilirubin to urobilin—so long at least as the liver and bile passages remain sterile. Under such conditions the intestinal source of urobilin remains the only one.

The same fact holds true when there is blood destruction. Extensive hemolysis, sufficient in many instances to lead to tissue icterus, and in some cases associated with liver injury, is never attended by the appearance of urobilin in the bile, urine, or stools, so long as bile is prevented from reaching the duodenum. The extravasation of blood into the tissues does not lead to the excretion of the pigment under such circumstances, nor do infectious processes.

When bile flow to the intestine is going on, as in dogs intubated for the collection of but a fraction of the bile, and blood destruction is produced in one of the ways just mentioned, urobilinuria develops and there is an increase in the urobilin content of bile and stool (see charts). An especially pronounced urobilinuria occurs after toluylenediamine has been given (see Protocol III). During the period of increased blood destruction the curve of urobilin elimination in the urine closely follows both in time and intensity the curves of the increase of bilirubin and urobilin in the bile. Kühl[18] records similar

[18] Kühl, G., *Arch. exp. Path. u. Pharmakol.*, 1924, ciii, 247.

findings after the use of phenylhydrazine. A similar, though less definite relationship was found to exist after intravenous hemolysis (see Protocol IV). In all the cases in which blood destruction occurred or was induced, bile was reaching the intestine through the common duct; in all there was an increase in pigment output by the liver; and in nearly all there was a marked fall in the percentage of the circulating hemoglobin.

What brings about the appearance of urobilin in the urine under such circumstances? Liver damage might be invoked to explain it in some. Though this may, indeed, have been partly responsible in certain instances, it is significant that in every case there was an increase in the urobilin and bilirubin output of the liver, indicating that there can have been no profound impairment in the ability of the organ to excrete the two pigments. During the height of the blood destruction caused by toluylenediamine the liver was, in one instance, excreting eight times the usual amount of bilirubin.

Unexpected light was thrown upon the problem thus posed by the observation that the giving of large amounts of bile by mouth to healthy animals will cause urobilinuria. This happens in animals losing the total bile after intubation, as also in those losing but a fraction of it,—though, the amount of bile which must be fed to produce urobilinuria is larger under the circumstances first mentioned (see Protocols VIII and IX). In such instances, and especially when pure bilirubin has been fed, there can be no question of liver injury. Evidently the determining factor is the presence in the intestine of an unusually large amount of bile pigment. Taken together the facts lead to the conclusion that urobilinuria during increased blood destruction is a secondary manifestation of the polycholia consequent on the liberation of hemoglobin. The large output of bilirubin derived from the liberated blood pigment leads to a large formation of urobilin in the intestines, which is absorbed therefrom in amounts too considerable for all to be dealt with by the liver, with the consequence that part escapes into the general circulation to be removed by the kidneys and urine.

The well known increase in the urobilin content of the stools during the course of diseases involving blood destruction finds a simple exemplification in our observations (see Text-figs. 3, 4, and 6). But it

will be noted that the increase in the stools usually occurs a day or two after the appearance of urobilinuria. Often it is not found at all. As already remarked, the amount of urobilin in the stools is normally subject to great fluctuations. It increases during diarrhea, in the absence of hemolysis,[7] and lessens with constipation. Its value as a measure of increased blood destruction is thus greatly diminished. In our experiments changes in the urobilin quantity in the stool have been far less dependable and significant than the occurrence of urobilin in the urine.

The constant occurrence of urobilinuria during the more acute stages of pernicious anemia has repeatedly excited the attention of clinicians. In such connections the question has often been raised whether there may not be a biliary disturbance traceable to some special involvement of the liver in the disease process. But the ability of the liver of pernicious anemia to excrete large amounts of bilirubin is shown by the finding of tremendous amounts of fecal urobilin in these cases. The responsible factor would seem, from this evidence, to be an augmentation in the rate of red cell destruction, though it is true that van den Bergh,[19] and Broun and others[20] have frequently found hemoglobin, hematin, and bilirubin in the serum of patients with pernicious anemia, and in fatal cases, Peabody and Broun[21] have seen tremendously increased phagocytosis of red cells in the bone marrow removed at autopsy. Warthin,[22] before them, had described similar findings in the spleen, lymph, and hemolymph glands and concluded that "the poison of pernicious anemia stimulates the phagocytes . . . . to increased hemolysis (cellular hemolysis)." From the evidence of the experiments just discussed blood destruction alone would seem to be sufficient to account for the intense urobilinuria. The passage of increased amounts of bilirubin into the intestine leads to the formation and absorption of more urobilin than the liver, often siderosed and fatty, can handle; the pigment escapes complete removal from the portal stream; reaches the general circulation; and is excreted by the kidneys.

[19] van den Bergh, A. A. H., Der Gallenfarbstoff im Blute, Leiden, 1918.

[20] Broun, G. O., Ames, O., Warren, S., and Peabody, F. W., *J. Clin. Inv.*, 1924–25, i, 295.

[21] Peabody, F. W., and Broun, G. O., *Am. J. Path.*, 1925, i, 169.

[22] Warthin, A. S., *Am. J. Med. Sc.*, 1902, cxxiv, 674.

Urobilinuria has long been known to occur in lobar pneumonia. This, with the occasional icterus, points to a disturbance in pigment metabolism. Since the liver in fatal cases with jaundice and uro-bilinuria usually shows cloudy swelling and even necrosis, the uro-bilinuria has been taken by many to be a reflection of the biliary disturbance. But not only is there liver injury to account for the finding, but also some evidence exists of excessive blood destruction. Peabody and Broun[21] have described increased phagocytosis in the bone marrow from patients dying of lobar pneumonia. It may be recalled in this connection that we have found decided increases in the amount of bilirubin put out by the liver of dogs suffering from distemper (see Text-fig. 7). We would suggest that increased blood destruction may well be a factor in the causation of the urobilinuria of infectious diseases though, of course, liver damage must often play a large part.

Thus far the results of frank blood destruction only have been considered. But urobilinuria has also been found in another group of conditions, those characterized by the escape of blood into the tissues with a subsequent slow destruction of the red cells and ab-sorption of the resulting pigments.[2] As already stated we produced hematomas in a few intubated dogs, but the subsequent increases in bilirubin output were very slight, and urobilinuria was not found. On the other hand, this latter phenomenon occurred regularly after operation, in animals from which only a part or none of the bile was being drained. In such of these cases as were intubated there was always to be noted a postoperative increase in the bilirubin of the bile, referable without doubt to the absorption of pigment from the blood extravasate consequent on the trauma of operation. The as-sumption that the anesthetic caused some slight liver derangement in such cases, a factor lacking when artificial hematomas alone were pro-duced without it, will explain the difference in the urobilin findings.

How may one compare the urobilinuria of excessive blood destruc-tion with that following liver disease? In each instance it is evident that some or all of the pigment has escaped removal from the portal blood by the liver, in the one case because there is too much of it to handle; in the other because the cells are diseased and cannot handle it all. In liver disease the functional reserve is decreased or lost; in excessive blood destruction it is overstepped.

From the practical point of view analysis of the findings reported in this and the preceding paper permits of at least two inferences which may prove to have diagnostic worth. Liver damage as such is never responsible for a high urobilin content of the stool; the amount of this pigment tends, on the contrary, to be low, as would follow from the tendency to bile suppression. In such connection the duration and intensity of the urobilinuria may have considerable value. In acute and severe liver disease urobilin may be abundant in the urine, but only transiently; for it disappears practically as soon as bile ceases to flow into the intestine.[10] The urine thereafter contains only the bilirubin of the developing jaundice.

By contrast with the foregoing when there is sustained blood destruction without serious liver injury urobilinuria is likewise sustained. Bilirubin may also appear in the renal output, if there be secondarily some obstruction to bile outflow, as not infrequently happens on sudden hemolysis. During persistent, sustained blood destruction the urobilinuria varies in degree with the amount of blood destroyed (see Text-fig. 3, which exemplifies this strikingly).

SUMMARY.

Further evidence is presented, in addition to that of our previous papers, that the intestinal tract is, under ordinary circumstances, the sole place of origin of urobilin. So long as the biliary tract remains sterile the presence of the pigment in bile and urine is entirely dependent upon the passage of bile to the intestine.

Animals rendered urobilin-free by the collection of all the bile from the intubated, uninfected common duct, remain urobilin-free during and after extensive blood destruction caused by intravenous injections of distilled water, as also after reinjections of the animal's own blood, hemolyzed *in vitro*. No urobilin appears in the bile, urine, or feces of animals so intubated when blood destruction has been caused by sodium oleate, or by an agent, toluylenediamine, which damages the liver as well as the blood.

On the other hand, when bile flow into the intestine is uninterrupted, urobilinuria occurs during blood destruction caused in any of the ways mentioned and it parallels, both in severity and duration, the destructive process.

Merely increasing the amount of bilirubin within the intestines of healthy dogs by feeding urobilin-free bile, will lead to marked urobilinuria. The extravasation of blood into the tissues, resulting from the trauma of an operation for intubation of a bile duct, does not lead to urobilinuria in animals losing all of the bile after this operation, but may do so when only a small fraction of the bile is drained, while the remainder reaches the intestine as usual. The production of artificial hematomas, without operation, is not followed by urobilinuria, under the circumstances last mentioned, but merely by an increase in the bilirubin of the bile. The effect on the liver of the anesthetic employed during the intubation may be responsible for the difference in the two cases.

During the course of certain intercurrent infections affecting some of the intubated animals, notably distemper, there was a drop in the hemoglobin percentage of the circulating blood, accompanied by an increased output of bile pigment or further by urobilinuria, when the conditions were such that bile still reached the intestine. The findings pointed to increased blood destruction as a factor in the urobilinuria.

The evidence presented, taken with that of our previous papers, suffices to demonstrate, that urobilinuria, occurring during blood destruction, is primarily the result of an increased excretion of bilirubin from which, in turn, an unusually large quantity of urobilin is formed within the intestine. The liver fails to remove from the portal blood all of the latter pigment which is resorbed and consequently some of it reaches the kidneys and urine.

Our work has been carried out on animals with uninfected biliary tracts and livers, save for one case which has special mention. The influence of infection of the biliary tract on the place of formation of urobilin and the development of urobilinuria will be discussed in a succeeding communication.

THE BILIARY ASPECTS OF LIVER DISEASE.[*]

By PEYTON ROUS, M.D.

(From The Rockefeller Institute for Medical Research.)

Were the project not a vain one, I would ask your indulgence to consider the state of our knowledge in case the biliary secretion, instead of the renal, was voided directly to the outside of the body. If, that is to say, the physicians of past times, whom we see in the Dutch pictures holding up a flask to the light, had had in that flask not urine, but bile. Knowledge would have been interestingly different. There would have accumulated a huge clinical pathology of the bile. For this secretion has its suppressions and its cholureses, its cylinders and desquamations, its pathologic albumin and other strange substances, as has the urine. But things are as they are; and until the specialist, with his little tubes, converts our inside into an outside, most doctors will have to be content with perceiving biliary disturbance at one or several removes.

Now, how small a biliary disturbance can the clinician perceive? Circumstances alter that case very much. The liver is the most silent of organs if only a moderate fraction of its cells are in a healthy state. The ducts from more than three-fourths of it can be abruptly obstructed, without causing even a ripple on the clinical surface of things.[1] Herringham[2] has collected a considerable number of cases in which, as a result of gradual obstruction of the left branch of the hepatic duct by a stone, the entire left lobe of the liver had atrophied. The patients in these cases never knew anything had happend to them. There was at no time any jaundice, and the condition was first recognized at autopsy. In such cases there had taken place, of course, a gradual compensatory hypertrophy of the portion of the hepatic tissue with unobstructed ducts. Liver cells have an astonishing ability to proliferate. After destruction of one-half of the hepatic tissue of dogs by chloroform, restoration may be complete within seven to nine days.[3]

[*] Read before the Association of American Physicians, May 5, 1925.

The functional causes for biliary disturbance offer a far more complex problem than do mechanical ones. While the liver works extremely well, is a more than adequate mechanism, each cell being capable of doing at least four times the work in biliary excretion that it ordinarily performs, yet it is insensitive. It lets bile pigment slip past, never ridding the blood entirely thereof. It is, in fact, a threshold organ for this pigment, like the kidney; and there are natural and unnatural thresholds of bilirubin removal from the blood stream as in the renal instance. One may well believe that there is an unnaturally high threshold in congenital hemolytic jaundice and, perhaps, in pernicious anemia. During Frerich's time, sixty years ago, jaundice was frequently ascribed to functional causes that in the present day we would rule out of court. If a person had a fit of temper or another emotional shock he might turn up all yellow next day. With a more critical scrutiny of these cases they have disappeared. But one type of functional icterus, namely, that due to fasting, does remain. There can be no doubt that when the normal individual has been deprived of food for some hours bilirubin increases in his plasma. The state of affairs is subicteric, as in many other conditions. "Bilious" would be a better word, were it not in such bad repute.

You will note that by degrees I have come to talk of bilirubin and jaundice, not of biliary constituents generally. It is natural to think in terms of the bile pigment, for we know relatively little of the phenomena involving bile salts and cholesterol. Methods of quantitating these substances are difficult, and the results uncertain. Certain one can be, however, that the cholates play a far more important role, both normally and in disease, than does bilirubin, and that many more gall stones are formed out of cholesterol than out of bilirubinate. Bile salts are the substances responsible for most of the injury during biliary disturbances. They are, furthermore, a specific product of the hepatic parenchyma, whereas bilirubin is not. The amount of cholesterol in the bile can be greatly altered by the character of the food, as McMaster has proven definitely.[4]

It is customary to inquire, when one sees a case of jaundice, whether the coloration is obstructive or hemolytic in derivation. The distinction has clinical uses, though, of course, all jaundice is blood-de-

rived.   We now know, thanks to the work of Mann and his associates,[5] that the formation of bilirubin from hemoglobin is almost, if not quite wholly, extrahepatic.   But the liver hallmarks the pigment as it puts it out,[6] with result that it can be recognized from that upon which the organ has not acted.   There is a remarkably close relationship between current blood destruction and the intensity of a simple obstructive jaundice.[7]   Under normal circumstances the variations in the amount of hemoglobin circulating as blood from day to day find a direct, if not entirely accurate, expression in the amount of bilirubin discharged into the bile.   The corpuscular wastage would seem to be recorded almost immediately in terms of bilirubin; the greater the number of cell deaths the more the bilirubin put out. When obstruction is produced, most of the bile pigment formed is retained within the body, and, other things being equal, the jaundice that ensues will be more intense when the animal is full blooded than when it is anemic.   If it is bled the jaundice diminishes very nearly in proportion to the amount of hemoglobin withdrawn only increasing again as the loss of blood pigment is repaired.

The intercurrent variations in hemoglobin and bilirubinemia during uncomplicated obstructive jaundice accord with each other so closely from day to day that it is evident bile pigment cannot be readily distributed to the tissues, else they would take up the slack, so to speak, of the bilirubin accumulation in the plasma, and there would be little diurnal variation therein.   To test the state of affairs is an easy matter.   On collecting a specimen of lymph from the leg of a long-jaundiced animal, one finds that it contains a negligible quantity of bilirubin as compared with plasma procured at the same time. Evidently there exists a barrier to the passage of bilirubin from the blood.   Tissue icterus is in point of fact only the imperfect, secondary, expression of a condition primarily confined to the blood pool.   Its intensity is, however, conditioned to no small extent by the tissue state.   Schürer[8] and Jädassohn[9] have recently described a singular condition which the former terms "icteric skin writing."   In their cases urticaria occurred, or was induced by artificial means, in patients that were jaundiced.   The urticarial wheals were far more deeply pigmented than the surrounding tissue.   In some instances this was due merely to the accumulation in the wheals of lymph colored like

that elsewhere. But in others more pigment had come through locally from the blood stream, and there was plainly an increased permeability of the vessel walls to bilirubin. The question is worth proposing whether an increased permeability of the vessels may not have a share in the development of fulminant icterus.

If jaundice is fathered by the blood then certainly it is born of the liver. In asking whether it is hemolytic or obstructive, one is really asking, does the blood break down so fast that the liver gets clogged secondarily, or is the liver so clogged to begin with that the products of normal blood destruction can find no outlet?

Pathologic disturbances affecting the ability of the liver cells to excrete bile pigment are a frequent cause of jaundice. The book written by Brulé[10] is instructive in this relation, and expresses well the French point of view as against that of a German school—the Germans are the metaphysicians of medicine—which would relegate all jaundice to mechanical causes. That there must be an icterus referable to cellular disturbances I believe most clinicians will agree. How else is one to explain dissociated jaundice, or the retention of two kinds of bilirubin, the so-called hemolytic and obstructive kinds? And how explain those cases in which, after operative drainage of the common duct, the bile, at first normal to all appearance, becomes lighter and lighter, though still copious, until finally a "white bile" is formed and the patient shortly dies with a greatly diseased liver?

It is simple to demonstrate the occurrence of jaundice from parenchymal disturbance by producing liver injury with chloroform.[11] As the injury develops the bile becomes progressively lighter and lighter, and shortly "white," while at the same time jaundice puts in an appearance. The secretion is not glairy, of the sort that would cause obstruction, but watery and clear. There are no changes within the hepatic tissue, such as would cause obstruction. It is probable that the partial or complete rejection of bile pigment by functionally damaged hepatic cells will explain many of the recorded instances of anomalous van den Bergh reactions.

There is another pigment, urobilin, which is important in relation to biliary disturbances. It is not an essential constituent of the bile, but is derived secondarily from the bilirubin of the secretion, and is hence directly dependent upon it. As a recent writer has

stated, clinicians believe urobilin (under which name urobilinogen may be included) to be formed in the intestines by the action of bacteria, resorbed in part from the gut, carried to the liver by the portal circulation, and, under normal circumstances, removed therefrom by this organ. The clinical belief is correct, as the recent work of McMaster and Elman clearly shows.[12]

Their experiments have proved that the history of the pigment is as follows: It is formed in the intestine from bilirubin by bacterial action, is resorbed in part, and ordinarily the resorbed portion is so completely taken out of the blood by the liver that only a trace reaches the urine. Of the portion taken by the liver some, perhaps all, is excreted into the bile, and hence the normal bile regularly contains urobilin. If, now, bile be diverted from the intestine so that no bilirubin reaches the gut, to be worked upon there by bacteria, the diverted bile soon comes to be free from urobilin; while if bile is prevented from reaching the intestine by ligation of the common duct urobilin soon disappears from both feces and urine. No matter how greatly one damage the liver of the animal with obstructed common duct, or with a fistula through which the bile is lost, the excreta remain free from urobilin The same holds true even when a large part of the blood is broken down within the body by experimental means.

The liver has not so large a margin of safety for urobilin as it has for bilirubin. If one tie off only a small twig of the hepatic duct some of the urobilin absorbed from the gut passes through the injured portion of the liver and is excreted into the urine. As result of relatively slight hepatic injury one has urobilinuria, whereas a much larger one would not result in bilirubinuria.

It would seem, from what has thus far been said, that urobilin might be relied on as a highly useful sign of liver injury. Unfortunately there is more to the story of it. We have been considering what happens under conditions when there is no infection. But when the liver is infected, things may be very different. If one obstruct the common duct after infecting the bile in the passages with urobilin-producing organisms, urobilinuria will occur.[13] Many people have supposed that the liver cells themselves can form urobilin, but the evidence speaks conclusively against this. It is bacteria

ensconced within infected ducts and perhaps within parenchyma, bacteria of a very special sort, which form the pigment out of bilirubin. To determine under what circumstances urobilin-producing organisms are found within the human liver will be an interesting task for the future.

Many of the bile constituents besides urobilin are resorbed in greater or less part from the intestine—bilirubin, cholesterol and, notably, the bile salts. The question may be asked, Is this resorption purposeful, brought about by a special arrangement? I do not believe that it is. Hinman[14] has shown that when the ureters are introduced into the small intestine by operative means, with result that all the urine is discharged into the gut, the animal resorbs the urinary constituents so completely as to die of uremia. It is probable that a similar process, incident to the physiologic activities of the mucosa of the gut, will suffice to explain the passage back into the organism of the bile constituents. Whipple[15] has trenchantly pointed out what would happen in case bile salts were quantitatively resorbed. With new salts being formed every day, and the old conserved through resorption, the patient would soon come to be, as he says, a pillar of bile salts. Granting all this, there can be no doubt that the body has adjusted itself to the resorption of biliary constituents so far as these influence its daily processes. Resorbed bile salts are known to stimulate bile secretion and for such reason are said to act as a "pace maker." In this relation it will not be amiss to point out that the bile obtained from a fistula animal is very different from the normal secretion. Owing to lack of the pace-maker influence the quantity secreted is greatly less than normal. Mc-Master and Elman[16] have recently intubated small ducts from the liver, while allowing the greater part of the bile to flow to the intestine, and they find that the secretion collected from day to day under such circumstances is about three times as copious as that one would get from the same liver portion if all of the bile were being diverted from the gut.

Biliary disturbances mean disturbances of other functions as well as of the biliary function itself. On prolonged obstruction bile salts cease to be formed. Some substances which should be taken up by the liver and excreted into the bile are no longer so excreted. The

hepatic cells form those curious little bodies known as bile thrombi which are not, as many have thought, inspissated bile, but a mixture of lipoids and protein, a product of damaged cells. The hepatic tissue does not take up hemoglobin with anything like the normal avidity. It does not lay down glycogen as well, and the quantity of sugar in the blood is increased. It does not form hippuric acid as well on test. In all these functions the organ obviously suffers, and in many others of which we know little. Yet it carries on remarkably well. I am speaking now of cases in which there is frank obstruction. When, by contrast, the liver parenchyma is at fault recovery or death takes place within a short period usually. Those cases in which one sees a long-standing icterus, with an excretion of bile still going on into the intestine, are cases in which the liver has been forced to the wall by repeated, localized injury, so that there has come to be less hepatic tissue than will suffice for the excretion of the daily quota of biliary constituents. This sort of thing happens at a late stage in Laennec's cirrhosis.

Recently there has been a productive revival of interest in the bile. Until it began our conception of biliary matters had been a masterpiece of the antique. Workers since Quincke had merely dusted off this masterpiece. But now, within a few years, much has come to be known. Clinical attention has been aroused, however, not by the new learning, but by the development of methods for quantitating the amount of bile in the blood, for obtaining bile from the duodenum and by the attempts to induce the gall bladder to discharge its contents. The question arises whether, when these technical advances have been perfected, and their limitations defined, clinical interest in the bile will flag? I do not believe it will, for the reason that workers are beginning to test liver function successfully. Not a few of the tests advocated are carried out with substances which find their way into the bile. In using these substances it is essential to know whether one is measuring liver function generally or merely biliary function. Before the point is settled much will inevitably be determined of biliary problems, and that is fortunate, because the bile is not merely an excretion—it is a highly purposeful secretion, and one susceptible of further utilization as an indicator of the liver state.

REFERENCES.

1. McMaster, P. D., and Rous, Peyton: Jour. Exper. Med., 1921, **33,** 731.
2. Herringham, W. P.: St. Bartholomew's Hospital Reports, 1905–1906, **41,** 15.
3. Davis, N. C., Hall, C. C., and Whipple, G. H.: Arch. Int. Med., 1919, **23,** 689.
4. McMaster, P. D.: Jour. Exper. Med., 1924, **40,** 25.
5. Bollmann, J. L., Mann, F. C., and Magath, T. B.: Am. Jour. Physiol., 1924, **69,** 371.
6. van den Bergh, H.: Der Gallenfarbstoff im Blute, J. A. Barth, Leipzig, 1918, p. 85.
7. Rous, Peyton, and Drury, D. R.: Jour. Exper. Med., 1925, **41,** 601.
8. Schürer, J.: Deutsch. med. Wchnschr., 1922, **48,** 593.
9. Jädassohn, J.: Deutsch. med. Wchnschr., 1923, **49,** 1544.
10. Brulé, M.: Recherches récentes sur les ictères, Masson et Cie, Paris, 1919.
11. Drury, D. R., and Rous, Peyton: Jour. Exper. Med., 1925, **41,** 611.
12. Elman, Robert, and McMaster, P. D.: Jour. Exper. Med., 1925, **41,** 503; McMaster, P. D., and Elman, Robert: Jour. Exper. Med., 1925, **41,** 513; McMaster, P. D., and Elman Robert: Jour. Exper. Med., 1925, **41,** 719; Elman, Robert, and McMaster, P. D.: Jour. Exper. Med., 1925, **42,** 99.
13. McMaster, P. D., and Elman, Robert: Jour. Exper. Med. (in press).
14. Hinman, F., and Belt, A. E.: Jour. Am. Med. Assn., 1922, **79,** 1917.
15. Whipple, G. H.: Physiol. Rev., 1922, **2,** 440.
16. McMaster, P. D., and Elman, Robert: Loc. cit.

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

IV. CONCERNING THE ONENESS OF THE BACTERIOPHAGE.

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D'Hérelle believes that the bacteriophage is a filterable, ultramicroscopic virus, parasitizing upon and destroying actively growing, susceptible bacteria. According to his conception, "there is but a single bacteriophage, common to both man and animals, capable by adaptation of acquiring a virulence toward all bacterial species."[1]

Evidence exists against this view. Some investigators, having failed in their attempts at adaptation of the bacteriophage,[2,3] believe in the existence of a number of serologically distinct strains, even among closely related varieties of the lytic principle.[4-8] This conception finds support in the fact that the secondary overgrowth of bacteria, appearing in the culture in the presence of lytic principle, is specifically resistant to lysis by the agent conditioning its appearance, but is often susceptible to the lytic action of another closely related lytic filtrate.[2, 5, 9, 10] Further evidence in favor of a multiplicity and relative specificity of lytic principles is to be seen in the differences in the characteristic size and appearance of plaques, or sterile spots,

[1] d'Hérelle, F., The bacteriophage. Its rôle in immunity, English translation by Smith, G. H., Baltimore, 1922, 121.

[2] Matsumoto, T., *Wien. klin. Woch.*, 1923, xxxvi, 759.

[3] Otto, Munter, and Winkler, quoted from Otto, R., and Munter, H., *Ergebn. Hyg., Bakt., Immunitätsforsch. u. exp. Therap.*, 1924, vi, 41.

[4] Wagemans, J., *Arch. internat. pharmacod. et thérap.*, 1923, xxviii, 181.

[5] Gratia, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 821.

[6] Seiffert, W., *Z. Immunitätsforsch., Orig.*, 1923–24, xxxviii, 301.

[7] Bail, O., and Watanabe, T., *Wien. klin. Woch.*, 1922, xxxv, 169.

[8] Bruynoghe, R., and Appelmans, R., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 96.

[9] Bail, O., *Z. Immunitätsforsch., Orig.*, 1923–24, xxxviii, 57.

[10] Okuda, S., *Arch. Hyg.*, 1923–24, xcii, 109.

when bacteria are grown in the presence of different lytic filtrates on the surface of agar.[10, 11]

In the course of experiments preliminary to a study of the influence of hydrogen ion concentration on the adsorption of lytic principle by bacteria, we have made observations which, in our opinion, are favorable to this view of a multiplicity of the lytic principle. It was found that the four lytic filtrates which were examined showed distinct differences in the response to changes in the hydrogen ion concentration of the medium, irrespective of the species of bacteria serving as substratum for the production of the active filtrates.

The experimental procedure was as follows:

Influence of Temperature and of Duration of Exposure.

Lytic filtrates, prepared by growing susceptible bacteria in the presence of active principle upon unbuffered broth, adjusted to pH = 7.4, were diluted 1:1000 in physiological salt solution,[12] and 0.2 cc. of this dilution was added to a series of tubes, each containing 1.8 cc. of buffer solutions, varying from pH = 2 to pH = 12 (thus diluting the original filtrate 1:10,000). These mixtures were kept at a constant temperature, and after a stated period of time 0.1 cc. of the contents of each tube of the series (containing now 10^{-5} cc. of the original filtrate) was transferred to 9.9 cc. of sterile buffered broth (pH = 7.4), and titrated by the serial dilution method.[13, 14]

Electrometric measurements have shown that the addition of 0.2 cc. of diluted filtrates to 1.8 cc. of the various buffers did not change appreciably their respective hydrogen ion concentrations. Similarly it was established that the subsequent transfer of 0.1 cc. of the mixture of various buffer solutions containing the lytic principle into 9.9 cc. of buffered broth (pH = 7.4) resulted, to all intents and purposes, in the immediate neutralization of solutions added.[15]

In the study of the adsorption of lytic principle by bacteria, to which this series of experiments was a preliminary, the tests were carried out at a temperature of +7°C., in order to avoid the rapid multiplication of bacteria and a corresponding increase in the concentration of the lytic principle during incubation. All the experiments reported in the present paper were performed at this tem-

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[11] Watanabe, T., Z. Immunitätsforsch., Orig., 1923, xxxvii, 106.

[12] Dilution was resorted to in order to shorten the subsequent titration series.

[13] Appelmans, R., Compt. rend. Soc. biol., 1921, lxxxv, 1098.

[14] Werthemann, A., Arch. Hyg., 1922, xci, 255.

[15] In the case of the extremely acid (pH = 2) and extremely alkaline (pH = 12) mixtures, the addition of 0.1 cc. of these mixtures to the 9.9 cc. of broth caused a drop or rise of the pH of the medium of less than 0.2.

perature ($+7°$C.). However, we carried out one of the experiments at both $+7°$C. and 37°C., in order to ascertain the effect of higher temperature on the rate of deterioration of the lytic principle at different pH. In the experiment mentioned lytic filtrate was allowed to remain in contact with various buffers, at 7°C., and at 37°C., for 3, 6, and 24 hours respectively. At the end of each period a sample (0.1 cc.) was taken from each mixture and titrated by serial dilution.[13, 14] The results of the titrations were first read at the end of 24 hours incubation at 37°C.; then again at the end of a further period of 24 hours of incubation. At this time the lytic titer in each of the tubes which received originally one or more active lytic units would have reached its maximum. For this reason, the tubes in which the occurrence of lysis was doubtful were heated for 30 minutes at 56°C., in order to destroy the resistant bacteria, and now 0.1 cc. of the contents of such tubes was transferred into a fresh tube containing 10 cc. of sterile broth, seeded with 0.1 cc. of an emulsion containing 1,000,000,000 susceptible bacteria per cc., and incubated for 3 to 4 hours at 37°C. At the end of this time, and before any overgrowth had taken place, the final readings were made.

As an illustration, results of the experiment at 7°C. are shown in Protocol 1. The sign $+$ in this protocol signifies the presence of lysis, as judged by absence or slight amount of visible growth, and the sign $-$ signifies apparent absence of lysis, or presence of bacterial growth equal or nearly equal to that occurring in a control tube of broth receiving 0.1 cc. of bacterial suspension only.

It will be seen from this protocol that direct readings of the results of titration, after 24 or 48 hours, were somewhat confusing, owing to the fact that the overgrowth of resistant bacteria often masks the coincident lysis of susceptible individuals in the culture. It is for this reason that a final test, by means of a transfer of 0.1 cc. from each tube (after heating) was resorted to, as stated above.

The results recorded in the protocol represent quantitatively the changes in activity of the Laudman Shiga lytic filtrate, which took place after its exposure to various buffers for 3 hours at $+7°$C. Identical titrations were performed after a period of 6 and 24 hours contact of filtrate with buffers at 7°C., as well as after a contact for the same periods of time at 37°C. The results of all these titrations are presented graphically on Chart 1, in terms of the minimum amount of the original mixture (Protocol 1, A) which contained at least one active unit of the lytic principle after the exposure to buffers for a fixed length of time, at a fixed temperature.

Protocol 1.

Effect of the Hydrogen Ion Concentration on Activity of the Lytic Filtrate Laudman Shiga at +7°C.

A

Laudman Shiga filtrate diluted 1:1000, cc...	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Buffer solutions, cc...	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
pH of buffer solutions (approximate)...	2.0	3.0	4.0	5.0	6.0	7.4	8.6	9.8	11.0	12.0
Resulting pH of mixture (electrometric)...	2.05	3.1	4.1	5.1	6.15	7.4	8.77	9.75	11.25	12.07

Mixed.   Left in ice box.   Samples taken after 3 hrs.

Amount of sample, cc...	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Buffered (pH = 7.4) broth, cc...	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9
Resulting pH (electrometric)...	7.25					7.4		7.4	7.6	7.4

1 cc. of the contents of each tube, after thorough mixing, transferred into 9.0 cc. of broth, and 1 cc. from that tube into 9.0 cc. of broth in the next series, and so on.

	Absolute amount of the original lytic filtrate present.	Amount of Mixture A present.
	cc.	cc.
Results of titration after 3 hrs.	1 × 10⁻⁵	0.1
	1 × 10⁻⁶	0.01
	1 × 10⁻⁷	0.001
	1 × 10⁻⁸	0.0001
	1 × 10⁻⁹	0.00001
	1 × 10⁻¹⁰	0.000001
	1 × 10⁻¹¹	0.0000001
	Control.	0.0

(Results columns for each pH series: 24 hrs., 48 hrs., Final transfer.)

* Occasional activity in this dilution was due to particulate nature of distribution of the active principle (see earlier paper: Bronfenbrenner, J. J., and Korb, C., J. Exp. Med., 1925, xlii, 483).

Comparative Resistance to Changes in Hydrogen Ion Concentration.

As can be seen from the chart, the destruction of the lytic power of the filtrate is considerably greater at 37°C. than at 7°C. So far as the effect of the length of exposure is concerned, in either case practically the entire change takes place very early, so that very little fur-
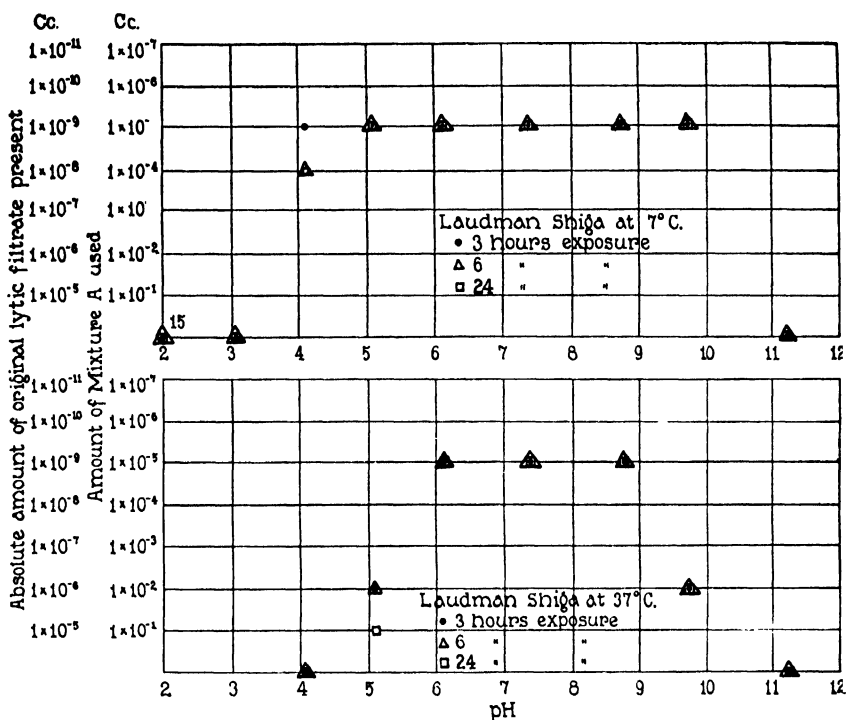
CHART 1. Effect of changes in the hydrogen ion concentration on the activity of lytic filtrate. On this as well as on the two other charts the points plotted on the base line indicate that there was no lysis in the largest amounts used (1 × 10⁻¹).

ther deterioration can be noted after the lapse of the first 3 hours. For the reasons already stated, in all further experiments the exposure of lytic filtrates to different buffers was carried out at 7°C. only, and, because of the above findings, the exposure in all cases was limited to 3 hours.

Using the procedure described, we have studied the resistance to changes in the hydrogen ion concentration of lytic filtrates active

respectively against *Bacillus coli*, *Bacillus dysenteriæ* Shiga, *Bacillus pestis caviæ*, and staphylococcus. Since the protocols of these experiments are in every detail similar to Protocol 1, further protocols have been omitted, and the findings have been recorded in Chart 2, in the manner identical with that used for construction of Chart 1.

Effect of "Adaptation" on the Characteristic Resistances.

The data plotted on Chart 2 have been obtained repeatedly, thus indicating that the various lytic agents studied have individual curves
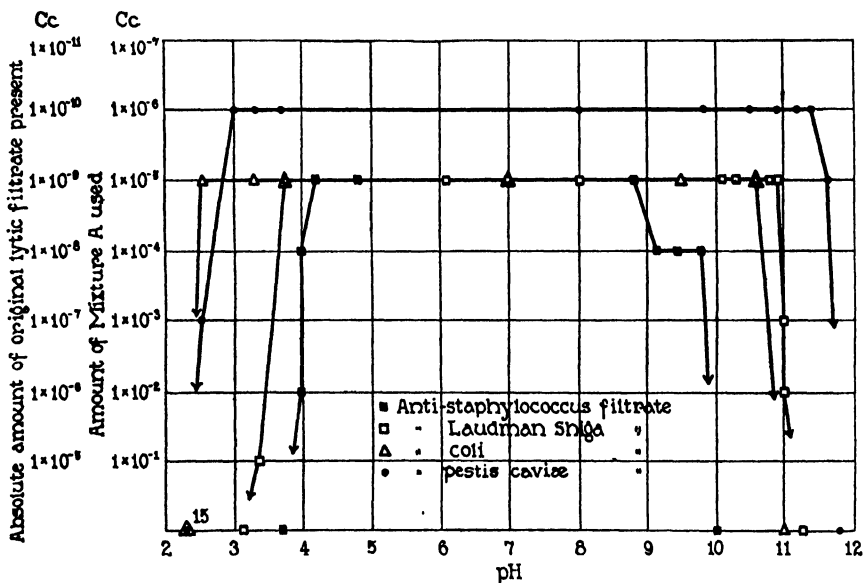
CHART 2. Comparative resistance of different lytic filtrates to changes in hydrogen ion concentration.

of resistance to changes in hydrogen ion concentration. The lytic agents active against *Bacillus pestis caviæ* seem to be the most resistant, remaining unaffected in the wide zone between pH = 3 and pH = 11.45. The lytic agent active against staphylococcus, on the other hand, is the least resistant, its resistance being limited by the hydrogen ion concentration corresponding to pH = 4.2 on the acid side, and pH = 8.8 on the alkaline side. The lytic filtrates active against *Bacillus coli* and *Bacillus dysenteriæ* Shiga resist alkalinity in almost the same degree, the limiting alkalinity for the former being pH = 10.6 and for

the latter pH = 10.9.   On the acid side the lytic agent active against *Bacillus coli* is limited by pH = 2.55, whereas that active against *Bacillus dysenteriæ* Shiga deteriorates beyond pH = 3.75.

If one accepts d'Hérelle's conception that there is only one lytic principle these differences would have to be explained as resulting from "adaptation" of the virus to different "hosts."

In order to determine whether the change in bacterial species serving as substratum for the production of lytic filtrate is sufficient to cause the change in its resistance to acidity or alkalinity, the following experiment was undertaken.
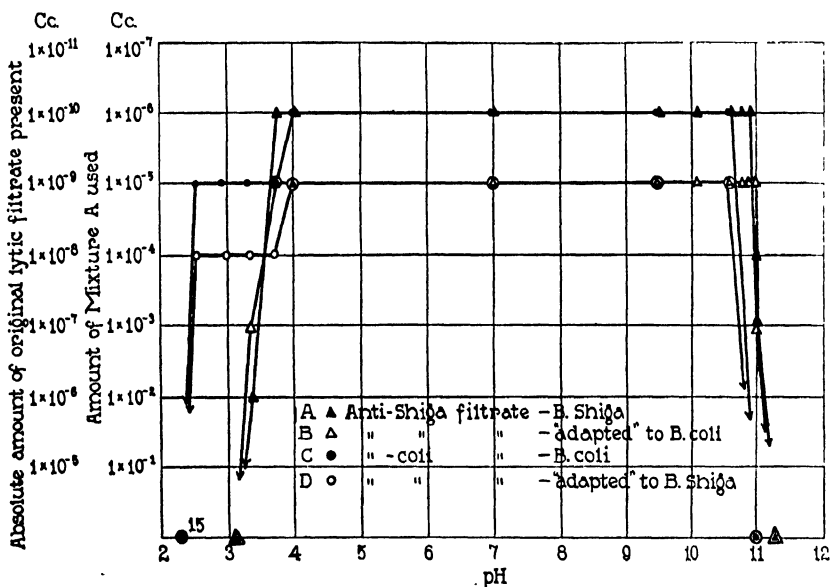
CHART 3.   Effect of "adaptation" on resistance of lytic filtrates to changes in hydrogen ion concentration.

Starting with a polyvalent lytic principle, acting primarily against *B. dysenteriæ* Shiga and secondarily against *B. coli*, two lytic filtrates were prepared; one (*A*), by passage through a number of generations upon a culture of *B. dysenteriæ* Shiga, and the other (*B*) upon a culture of *B. coli*.   The number of passages (ten) was amply sufficient to have eliminated all traces of activity due to the original filtrate.   At the end of these passages the filtrates thus obtained caused lysis of the respective bacteria when present in culture in the amounts of 10^{-10} and 10^{-9} cc. respectively.

In a similar manner two filtrates were prepared, starting with another polyvalent

bacteriophage, active principally against *B. coli*, though causing also a less complete lysis of Shiga bacillus. In this case one filtrate represented the result of many passages on a culture of *B. coli* (*C*), and the other ten passages on Shiga bacillus (*D*).

All four filtrates were diluted with buffer solutions at various pH and titrated after exposure to them for 3 hours at 7°C., exactly as before. The results of these titrations have been plotted on Chart 3.

Although the initial titers of both the filtrates of the "adapted" bacteriophages (*B* and *D*) were somewhat lower than those of the respective original ones (*A* and *C*), nevertheless the resistance to changes in hydrogen ion concentration was identical for each original and its adapted material.

SUMMARY AND CONCLUSIONS.

Lytic filtrates, active against *Bacillus dysenteriæ* Shiga, *Bacillus coli*, *Bacillus pestis caviæ*, and staphylococcus respectively, proved to be differently affected by changes in hydrogen ion concentration.

Anti-staphylococcus lysin was the least resistant of the four, showing deterioration in 3 hours at 7°C. beyond the zone of hydrogen ion concentration limited by $C_H = 6.3 \times 10^{-5}$ and $C_H = 1.6 \times 10^{-9}$. Under the same conditions, the zone of resistance of anti-*coli* filtrate lay between $C_H = 2.7 \times 10^{-3}$ and $C_H = 2.5 \times 10^{-11}$, and that of anti-Shiga between $C_H = 1.7 \times 10^{-4}$ and $C_H = 1.3 \times 10^{-11}$. Anti-*pestis caviæ* filtrate was most resistant of the four, retaining its full activity in the zone from $C_H = 1 \times 10^{-3}$ to $C_H = 3.5 \times 10^{-12}$.

The fact that these differences in individual resistance persisted, notwithstanding the repeated passage of lytic filtrates through cultures of bacteria other than those against which they were primarily active, seems to offer evidence in favor of a multiplicity of bacteriophages.

SEROLOGICAL STUDIES ON THE BLOOD OF THE PRIMATES.*

I. THE DIFFERENTIATION OF HUMAN AND ANTHROPOID BLOODS.

BY K. LANDSTEINER, M.D., AND C. PHILIP MILLER, JR., M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The problem of man's kinship to his closest relatives in the animal kingdom has been studied by the methods of comparative anatomy and paleontology and the results of these investigations are developing into a special branch of learning. When serology provided a new technique for recognizing the biochemical properties which characterize species it became inevitable that this technique should be applied to the problem of man's ancestry.

The serological procedure which has been chiefly followed is that of the precipitin reaction.

Grünbaum[1] made the first experiments with anthropoid blood. He found that anti-human serum gave a precipitate with the blood of the gorilla, the orangutang, and the chimpanzee "practically indistinguishable from that obtained with human blood either in quality or quantity. Occasionally it has seemed that the blood of the orang gave a more gelatinous precipitate as compared with the granular precipitate of the other bloods, but this may have been due to accidental circumstances." He prepared immune sera against gorilla, orang, and chimpanzee bloods and was "unable to assert that there is any difference of reaction amongst the many combinations of anti-serum and blood which can be made with the four above-mentioned bloods and sera." In consideration of these conclusions it seems difficult to interpret the apparently specific reactions observed by him microscopically and mentioned incidentally.

The well known studies of Nuttall[2] on the precipitin reaction had for their object the correlation of the serological properties of the serum protein of

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* A preliminary communication has appeared in *Science* (Landsteiner, K., and Miller, C. P., Jr., *Science*, 1925, lxi, 492).

[1] Grünbaum, A. S. F., *Lancet*, 1902, i, 143.

[2] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

animals with their position in the zoological system. Using several anti-human sera and one each of anti-chimpanzee, anti-orang, and anti-baboon serum, Nuttall made qualitative tests on the serum[3] of a great number of animals, including primates.

With anti-human immune serum he obtained positive reactions with 100 per cent of the specimens of man and anthropoid apes examined, 92 per cent of the Old World monkeys (Cercopithecidæ), 78 per cent of the New World monkeys (Cebidæ), and 50 per cent of the marmosets (Hapalidæ). He found that the occurrence of positive reactions was directly proportional to the proximity to man as indicated by the usual criteria of classification.

Nuttall also made a smaller series of tests in which he estimated the volume of precipitate. With chimpanzee serum the volume was 130 per cent of that obtained with human serum, but the precipitate was less compact; with gorilla serum, 64 per cent; with orang serum, 42 per cent; with mandrill serum, 42 per cent; with baboon serum, 29 per cent; and with the serum of one of the New World monkeys, 29 per cent. Nuttall remarks that the figure for the orang is probably too low. Since, however, in precipitin reactions the amount of precipitate has a maximum at a certain concentration of the antigen, the estimation of this amount at an arbitrary dilution does not exactly measure the activity of a given serum. No statement was made by Nuttall as to the possibility of differentiating with certainty human and anthropoid serum by the precipitin reaction.

According to Chiò[4] anti-human serum reacts equally well on human and orang-utang serum. His results with anti-monkey serum were likewise in agreement with Nuttall's. Friedenthal[5] also asserts that the serological differentiation of man and the anthropoid apes cannot be made by any method. He had previously[6] tried to investigate the relationship of species by observing the occurrence or non-occurrence of hemoglobinuria after heterologous transfusions. The existence of individual differences alone makes this method unreliable. He noted that an injection of 25 cc. of human blood into a chimpanzee produced no symptoms. Friedenthal also reported that the blood of lower monkeys was hemolyzed *in vitro* by human serum and human blood by monkey serum.

Marshall[7] made a more careful study of human and monkey blood by means of normal and immune hemolytic sera. He found that normal heterologous sera (goat, sheep, ox, goose, and rabbit) were practically equally hemolytic for human and *Macacus* blood. Human anti-erythrocyte immune sera hemolyzed the bloods of *Macacus rhesus* and *Macacus cynomolgus* almost as well as human bloods, while

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[3] In the original publications the term blood is commonly used since in many cases solutions of blood were used for the tests. The tests, however, do not involve the blood cells but the serum protein.

[4] Chiò, M., *Atti r. Accad. sc.*, 1905–06, xli, 1093.

[5] Friedenthal, H., *Arch. Physiol.*, 1905, 1.

[6] Friedenthal, H., *Arch. Physiol.*, 1900, 494.

[7] Marshall, H. T., *J. Exp. Med.*, 1901–05, vi. 347.

anti-monkey serum was only feebly hemolytic for the human bloods examined. Differences between the two kinds of blood were also found by absorption experiments. Nevertheless, Marshall concluded that "there is quite a close relationship between the corpuscles of human beings and both of the varieties of monkeys examined."

There has been a considerable amount of work done[8-13] on the differentiation of human and monkey proteins, especially with regard to the forensic identification of blood stains. The results agree with those of Nuttall in showing that anti-human sera react with monkey serum, but less intensely than with human serum. The specificity of the reactions can be increased by previous saturation with monkey serum.[14,15]

One observation by von Dungern and Hirschfeld[16] may be considered as indicating a difference between human and chimpanzee blood. These authors tested the action of human Group III serum after absorption with human Group II blood, on the red cells of one chimpanzee. The agglutinins of three such sera were completely removed for chimpanzee and for human blood. But one serum thus treated would still agglutinate chimpanzee blood and none of several human bloods. It is impossible to decide with certainty from this observation whether the findings bespeak a constant species difference. (See the following paper of this series, pages 128 and 130.)

We know of no additional work on the serological properties of the blood of anthropoids since the paper of von Dungern and Hirschfeld (1911).

It is possible that the precipitin technique can be improved so as to yield additional information on the interrelationship of the primates. Another method which suggests itself is the use of anti-erythrocyte sera. In spite of the great amount of work on hemolysis and hemagglutination, these reactions have never been systematically exploited in the study of species relationship as has the precipitin reaction. The explanation for this neglect lies apparently in the presumption that the species specificities of the precipitins and of the hemagglutinins are of the same order and that species specificity means protein specific-

[8] Wassermann, A., and Schütze, A., *Berl. klin. Woch.*, 1901, xxxviii, 187.
[9] Uhlenhuth, *Deutsch. med. Woch.*, 1902, xxviii, 659, 679.
[10] Biondi, C., *Vierteljährschrift gerichtl. Med.*, 1902, xxiii, suppl. 1.
[11] Layton, E. N., *Tr. Chicago Path. Soc.*, 1903, v, 217.
[12] Ewing, J., *Proc. New York Path. Soc.*, 1903–04, iii, 14.
[13] Stern, R., *Deutsch. med. Woch.*, 1901, xxvii, 135.
[14] Fujiwara, K., *Deutsch. Z. ges. gerichtl. Med.*, 1922, i, 754.
[15] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91.
[16] von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526.

ity.   It was tacitly assumed that the results in the two types of re-action would run parallel.   Recently it has been pointed out (Land-steiner and van der Scheer[15,17,18]) that this supposition is unwar-ranted.   As had been surmised before, the antigens which engender antibodies against red blood cells do not consist simply of proteins. It is possible to extract from erythrocytes by means of alcohol non-protein substances, possibly of a lipoid nature, which have binding and immunizing properties.   Certain inconsistencies regarding the antigens of red corpuscles have been reconciled.[17]

Considering the chemical dissimilarities of corresponding antigens, it is not surprising to find differences in the specificity of antibodies against the serum proteins and those against the red blood cells. One of the most striking and for our present purpose most important observations is this, that by the use of anti-erythrocyte immune bodies, even those present in normal sera, one can differentiate read-ily species so closely related that their sera are distinguishable only with difficulty by means of the precipitin reaction.[15,17]   We have therefore undertaken to study the blood cells of the anthropoid apes by means of anti-erythrocyte sera.

<div align="center">EXPERIMENTAL.</div>

Methods.—The two methods which we have mostly employed have been: first, the determination of the hemagglutinin titers of anti-erythrocyte sera on the blood cells of man and monkeys, and second, the determination of the fraction of agglutinin which remained after absorption by red blood cells of the various species.   The titrations were made in the usual way in series of tubes con-taining decreasing concentrations (by halves) of inactivated immune serum. Each tube contained 0.5 cc. of the solution to which was added 1 drop of a 2.5 per cent suspension of washed blood cells.   The readings were usually made after 1 or 2 hours at room temperature.   For the absorptions, the immune sera, each suitably diluted according to its strength, were mixed with one-half volume of packed, washed red cells and allowed to stand at room temperature (up to 2 hours), during which time they were occasionally mixed by inversion of the tubes, and then kept in the refrigerator overnight.   They were then centrifuged and the supernatant fluids titrated as described above.

The immune sera were prepared in the usual manner by a series of intravenous and intraperitoneal injections into rabbits of washed erythrocytes.   Anti-human immune sera were prepared against blood cells of Groups I, II, and III.

[17] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xli, 427; xlii, 123.

[18] Landsteiner, K., and van der Scheer, J., *J. Immunol.*, 1924, ix, 213.

Comparison of Human and Anthropoid Bloods.

Comparison of the titers of several anti-human immune sera for human and chimpanzee bloods respectively yielded no constant dif-

TABLE I.

Titration of Anti-Human Immune Sera with Human and Chimpanzee Erythrocytes.

		Cells.		
	Group.	Human.		Chimpanzee E.
		Group I.	Group II.	
		Highest effective dilution.		
Immune Serum 24...................	I	200	200	100
" " 26...................	I	500	250	375
" " 420..................	II	500	750	750
" " 421..................	II	3000	2000	2000

Reading after 2 hours at room temperature. Readings of all weak reactions were made microscopically in this as well as in the other experiments.

TABLE II.

Agglutination of Human and Chimpanzee Erythrocytes by Anti-Human Serum after Absorption with Chimpanzee Cells.

Anti-human erythrocyte serum 23 (Group I) (1/20 dilution) absorbed with cells of Chimpanzee F.

	33	34	35	36	37	38	39	40	18	25	28	8	W	5	6	7	8	9	10	11	12	13	Chimpanzee E.
Group.	I .	II	II	I	II	I	II	II	I	IV	I	III	II	I	I	I	II	IV	II	I	II	II	
	+±*	+	+±	+±	+	+	+	+	+	+±	+	+	+	+	+	+±	+	+	+±	+	+	+	0

Tests made in small tubes with the following quantities.

      1 drop absorbed immune serum.

      1 drop salt solution.

      1 drop 2.5 per cent suspension of washed blood cells.

Readings made after 2 hours at room temperature.

* In this and the following tables the signs have the following significance: 0 = negative; F. tr. = faint trace; Tr. = trace; ±, +, +,±, ++, etc.

ferences. Often the titers for chimpanzee blood were appreciably lower than for the human; but differences in the opposite direction

also occurred. The titers for human blood were also inconstant probably in part due to the difference in the blood group. The results of an experiment are shown in Table I. For other examples, see the absorption experiments below. Titrations with two anti-chimpanzee immune sera gave distinctly higher titers for the homologous bloods (see Table IV) in most cases.

In one experiment with orang blood the results were comparable to those with chimpanzee blood, while gibbon blood gave a definitely lower titer.

TABLE III.

Titration of Anti-Human Erythrocyte Serum after Absorption with Chimpanzee Cells.

Blood cells.	Anti-human erythrocyte serum 23 (Group I) (1/40 dilution).			Anti-human erythrocyte serum 344 (Group II) (1/20 dilution).		
	Control unabsorbed.	Absorbed with Chimpanzee E.	Absorbed with Chimpanzee F.	Control unabsorbed.	Absorbed with Chimpanzee E.	Absorbed with Chimpanzee.F.
	Highest effective dilutions.					
Chimpanzee E...............	750	<40	<40	320	<20	20
" F...............	1000	320	<40	320	80	<20
" H...............	750	40	<40	320	<20	<20
" I...............	500	<80	<40	320	<20	<20
Human 72 (Group I).........	4000	2560	640	1600	1280	320
" 77 (" II)........	2000	640	160	800	320	320
" 78 (" III).......	2000	1280	320	800	640	240
" L (" IV).......	2000	640	320	800	320	240

Reading after 1 hour at room temperature.

Absorption   Experiments.—Experiments   in   which   anti-human immune sera were absorbed with chimpanzee erythrocytes and *vice versa* yielded unequivocal and constant results. Such human bloods must be selected which will not leave behind any group-specific agglutinins. After removal of the agglutinins for chimpanzee blood, such liquids always contained agglutinins for human bloods of all groups. We have tested a great number (68) of human bloods in this manner since it became evident that considerable individual variation exists. The bloods of four chimpanzees were used. Table II is given as an example.

To determine the proportion of agglutinins in anti-human immune

serum which act on both human and chimpanzee bloods, and those which are specific for human blood alone, the supernatants of the absorbed sera were titrated. In consideration of the existence of individual differences in human bloods, the fluids were tested on a great number of blood specimens, especially selected for their dissimilarities. Table III shows the findings in a typical absorption experiment.

The tests show that in all cases with appropriate sera a considerable portion of the agglutinating capacity of the serum remained after absorption with the heterologous blood. This portion approximated in

TABLE IV.

Titration of Anti-Chimpanzee Immune Serum after Absorption with Human Blood Cells.

Serum 475 (1/10 dilution) absorbed with human erythrocytes (Group I).			Serum 474 (1/20 dilution) absorbed with human erythrocytes (Group II).		
Blood cells.	Control unab-sorbed.	Ab-sorbed.	Blood cells.	Control unab-sorbed.	Ab-sorbed.
Human (Group I)......	120	<10	Human (Group I)... ..	200	<40
" (" I)......	240	<10	" (" II)......	200	<20
" (" I)......	80	<10	" (" III)......	400	20
" (" I)......	120	<10	Chimpanzee E..........	400	120
" (" II)......	80	<10	" F..........	600	160
" (" III)......	60	<10			
Chimpanzee E..........	320	160			

Reading after 1 hour at room temperature.

the case of immune serum 344, 20 to 40 per cent after absorption with blood of Chimpanzee F; 40 to 80 per cent after absorption with blood of Chimpanzee E (see Table V). The fraction acting on both bloods was therefore between 20 and 80 per cent. It will be observed in Table III that individual differences were not limited to human bloods, for the blood of one chimpanzee showed a peculiarity. After absorption with the cells of Chimpanzee E as a rule the immune serum still contained an appreciable quantity of agglutinins for Chimpanzee F. This observation held true for this blood in a number of other tests. Absorption with the cells of Chimpanzee F, on the other hand, removed all of the agglutinins for all of the chimpanzee bloods tested, and in all instances left behind agglutinins for human blood. Analo-

gous experiments were made with several different immune sera. It is quite possible that similar results could be obtained with the serum of normal animals, but we have not made such experiments. Results confirming those reported were obtained when anti-chimpanzee immune sera were absorbed with human blood (see Table IV).

The conclusion to be drawn from these experiments is that it is regularly possible to differentiate human and chimpanzee bloods by means of suitable anti-erythrocyte immune sera.

As we had at our disposal only small quantities of orang blood only one absorption experiment was made on anti-human serum with orang blood. The absorbed liquid was insufficient for titrations, and the tests were made with single drops as in the experiments in Table II. The experiment demonstrated that orang blood differs from human blood and indicated that the relationship between orang and chimpanzee bloods is closer than it is between orang and human. Agglutinins remained for the eight human bloods tested and to a larger degree for that of Chimpanzee F, which was shown before to have an individual peculiarity, while all the agglutinins for the other two chimpanzees and four orangs were absorbed.

It has been claimed by Bruck[19] that the sera of various human races can be differentiated by the complement fixation reaction; but this has been denied by other authors.[20,21] We have examined by the methods employed in the other experiments the blood of negroes. Our experiments show that if serological differences do exist between the bloods of white men and American negroes—no longer a pure race—they are much smaller than those between man and the anthropoid apes. So far we have been unable to demonstrate any characteristic difference. It is not impossible, however, that slight differences might be found if individuals of several races preferably of pure blood were carefully studied by this method in all of its modifications.

Comparison of the Bloods of Man and the Anthropoid Apes with Those of the Lower Monkeys.

In another series of experiments we have studied the serological relationship of the erythrocytes of man and chimpanzee to those

[19] Bruck, C., *Berl. klin. Woch.*, 1907, xliv, 793.
[20] Marshall, H. T., and Teague, O., *Philippine J. Sc., Sect. B*, 1908, iii, 357.
[21] Fitzgerald, J. G., *J. Med. Research*, 1909, xxi, 41.

of the lower monkeys. Table V shows that immunization with the bloods of lower monkeys (baboon and macaque) did not engender any considerable quantity of antibodies against the blood of the higher apes, and *vice versa*. It should be noted that the sera employed in the absorption did not have a very high titer and that some previous experiments showed there was a somewhat greater fraction of agglutinins acting on both bloods in question (see references 7 and 18).

TABLE V.

Titration of Various Anti-Erythrocyte Immune Sera after Absorption with Various Primate Blood Cells.

Bloods.	Anti-human erythrocyte serum 344 (Group II) (1/20 dilution).			Anti-chimpanzee erythrocyte serum 474 (1/20 dilution).			Anti-baboon erythrocyte serum 458 (1/20 dilution).			Anti-macaque erythrocyte serum 456 (1/20 dilution).		
	Control unabsorbed.	Absorbed by baboon cells.	Absorbed by macaque cells.	Control unabsorbed.	Absorbed by baboon cells.	Absorbed by macaque cells.	Control unabsorbed.	Absorbed by human Group I cells.	Absorbed by chimpanzee cells.	Control unabsorbed.	Absorbed by human Group II cells.	Absorbed by chimpanzee cells.
Human (Group II)............	400	400	400	160	160	160	80	<20*	20	60	<20	40
Chimpanzee......	200	200		640	640	480	80	80	<20	40	20	<20
Baboon..........	80	<20	<20	40	20	<20	600	400	400	800	800	800
Macaque........	80	40	<20	40	<20	<20	400	300	200	800	800	800

Reading after 1 hour at room temperature.
*Test made with Group I blood.

When absorbed with monkey blood the agglutinating powers of both the anti-human and anti-chimpanzee sera were not markedly affected. A similar result was obtained on treating anti-baboon and anti-macaque sera with human and chimpanzee blood respectively. Also in this experiment a distinct difference between human and chimpanzee blood could not be demonstrated.

Comparison of the Bloods of the Lower Monkeys.

With the method employed above a difference was demonstrable between the two genera of Old World monkeys (Catarrhina) investi-

TABLE VI.

Titration of Anti-Baboon Erythrocyte Serum after Absorption with Macaque Blood Cells.

Serum 458 (1/20 dilution) absorbed with erythrocytes of *Macacus rhesus*.

Bloods.	Control unabsorbed.	Absorbed.
Human Group II..............................	80	80
Chimpanzee F................................	80	80
Baboon......................................	1000	320
Macaque.....................................	500	<20

Reading after 1 hour at room temperature.

TABLE VII.

Titration of Anti-Macaque Erythrocyte Serum after Absorption with Baboon Blood Cells.

Serum 456 (1/20 dilution) absorbed with erythrocytes of baboon.

Bloods.	Control unabsorbed.	Absorbed.
Human (Group I).............................	40	20
Chimpanzee E................................	40	20
Baboon......................................	1600	<20
Macaque.....................................	800	40

Reading after 1 hour at room temperature.

TABLE VIII.

Titration of Anti-Baboon and Anti-Macaque Immune Sera after Absorption with Sapajou Erythrocytes.

Blood cells.	Anti-baboon immune serum 458 (1/20 dilution). Absorbed with blood cells of sapajou (*Cebus hypoleucus*).		Anti-macaque immune serum 456 (1/20 dilution). Absorbed with blood cells of sapajou (*Cebus hypoleucus*).	
	Control unabsorbed.	Absorbed.	Control unabsorbed.	Absorbed.
Baboon......................	640	640	640	640
Macaque.....................	640	640	320	320
Sapajou.....................	80	<20	80	<20

Reading after 3 hours at room temperature.

gated, more especially by the use of the antiserum against the baboon in comparison with that against the macaque. This may have been due to an accidental circumstance (see Tables VI and VII).

The differences between the two species of Catarrhina and of one New World monkey—ringtail or sapajou monkey (*Cebus hypoleucus*)—were very marked. In this case the titration alone showed a striking specificity.

The absorption of the anti-baboon and anti-macaque sera with the blood of the sapajou did not diminish the titer for the cells of baboon and macaque (Catarrhina).

DISCUSSION.

The experiments described show that a definite and constant serological difference is demonstrable between the bloods of man and the two anthropoids studied—chimpanzee and orang-utang. The experiments also show that this method of agglutination by absorbed immune sera is suitable for the demonstration of serological differences between the bloods of species so closely related that they are indistinguishable by the precipitin reaction as it is usually employed. The difference between the bloods of the lower monkeys on the one hand and of man and the anthropoids on the other is considerably greater than that between the two latter, as is seen from the titers of the immune sera and the results of absorption experiments. The experiments permit of a further consideration. Both anti-human and anti-chimpanzee serum, after absorption with the blood of a lower monkey, do not lose an appreciable amount of their agglutinin content. Also anti-monkey sera behave quite similarly when treated either with chimpanzee or with human blood. It may be concluded, therefore, that anthropoid blood is not much more similar to that of the lower monkeys than is the blood of man to that of lower monkeys. This conclusion is in agreement with the accepted view of zoologists that man has not evolved directly from any of the existing species of primates, as was formerly supposed, but that the Catarrhina, anthropoids, and man have all sprung from a common stock. (See the diagrammatic representation in the third paper of this series, page 145.)

It is of interest to compare the degree of difference which we have

found between the bloods of man and chimpanzee with that already observed between the bloods of other pairs of closely related animals. In the case of the horse and donkey,[18] for instance, the proportion of homologous agglutinins left after absorption with the heterologous blood is the same or greater than in the case of man and chimpanzee, when one makes allowance for individual differences.   Measured by the agglutinin reaction bloods of the latter pair would seem to be no farther distant than those of the horse and donkey, between which hybridization is easily possible.   It must not be overlooked, however, that horse and donkey are regarded as considerably closer related morphologically than are man and apes.

SUMMARY.

While the precipitin tests do not differentiate, according to the results of previous workers, between the serum proteins of man and chimpanzee, a clear-cut differentiation between the blood cells of man and the anthropoids was obtained by means of hemagglutinins.

According to our tests on bloods of whites and negroes, constant racial serological differences among human bloods, if they exist at all, are certainly smaller than the differences between the bloods of man and the anthropoid apes.

The serological differences between man and the lower monkeys appear to be no greater than those between the anthropoid apes and the lower monkeys.   These findings confirm the opinion that the anthropoid apes do not rank in the genealogical tree between lower monkeys and man.

SEROLOGICAL STUDIES ON THE BLOOD OF THE PRIMATES.

II. THE BLOOD GROUPS IN ANTHROPOID APES.

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Knowledge of the blood groups in man[1] has assumed an anthropological significance as result of the work on their racial distribution by L. and H. Hirschfeld.[2] The studies of these workers, which have been confirmed and amplified by numerous others, have established the fact that the percentages of the four blood groups vary in the different human races. In substance it has been shown that among North Europeans Group II is predominant over Group III, while the opposite ratio holds for Asiatics and Africans. Other striking features are for instance a great preponderance of Group I among North American Indians (Coca and Deibert[3]) and a remarkably low percentage of Group I in Ainos.[4]

The intricate questions of the origin and racial distribution of the blood groups are of sufficient importance to anthropology and ethnology to make worth while the accumulation of all information which may give clues to their solution. An obvious question which presents itself is whether substances identical or similar to those which characterize serologically the human groups are present in the bloods of the other primates.

Observations on this point have been made by von Dungern and Hirschfeld.[5] They studied the blood cells of one chimpanzee. According to these results the

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[1] Landsteiner, K., Wien. klin. Woch., 1901, xiv, 1132; Oppenheimer's Handbuch der Biochemie, Jena, 1910, ii, pt. 1, 414.

[2] Hirschfeld, L., and Hirschfeld, H., Lancet, 1919, ii, 675.

[3] Coca, A. F., and Deibert, O., J. Immunol., 1923, viii, 487.

[4] Ninomiya, Y., Tôhoku J. Exp. Med., 1925, vi, 266. Grove, E. F., personal communication.

[5] von Dungern and Hirschfeld, Z. Immunitätsforsch., Orig., 1911, viii, 541, 547.

blood of this chimpanzee corresponds closely to the human Group II.   Absorption of three human Group III sera with human Group II erythrocytes removed all of the agglutinins for this chimpanzee blood.   But after similar treatment of a fourth serum a part of the agglutinins for the chimpanzee cells was left.   Two chimpanzee sera were also examined.   One of them acted on all human bloods of Group III and on some of Group II but on none of Group I.   The other serum contained heteroagglutinins for human bloods, besides group agglutinins for human blood of Group III.   If one assumes that the cells belonging to these chimpanzee sera possessed group-specific properties, one of them can be classified as Group II and the other as either Group I or II.

The blood cells of macaque monkeys did not react characteristically when treated with different human sera.

EXPERIMENTAL.

Methods.—The presence in human serum of heteroagglutinins for anthropoid bloods precludes the application to them of the simple routine technique for the grouping of human blood and renders the absorption tests with normal human sera less reliable.   We have therefore employed the isoagglutinin reaction only in connection with two other methods, both of which yielded wholly reliable results, while they have the advantage of being applicable to small amounts of blood, a consideration of some moment in the case of our material.

The methods by which the effect of the heteroagglutinins was obviated were: first, employment of purified "agglutinin solutions,"[6] and second, the use of group-specific immune sera[7,8]—described below as Methods B and C respectively.

A. For the simple isoagglutination tests 1 drop each of normal human serum, salt solution, and 2.5 per cent erythrocyte suspension were mixed in small tubes.

B. Preparation of the "agglutinin solutions:" 5 cc. of strongly agglutinating human serum, Group II or III, was mixed with 5 drops of packed, washed human blood cells of Group III or II, respectively, and allowed to stand about 2 hours at room temperature, during which time they were occasionally agitated.   The cells were then separated by centrifugalization and washed three times with salt solution.   The sediment, which still showed strong agglutination, was taken up in about 0.6 cc. of salt solution and placed in a water bath at 56°C., for not longer than 5 minutes, during which time it was shaken.   The clumps were usually broken up by this treatment.   The tube was then placed in a large centrifuge tube filled with water of 56°C. and centrifuged a short time to throw down most of the sedi-

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[6] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905.

[7] Landsteiner, K., *Wien. klin. Rundschau*, 1902, No. 40.

[8] Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419.

ment as quickly as possible.   The supernatant fluid was then removed and centrifuged a longer time unheated.   Tests with such agglutinin solutions were made in small tubes as described under A above.

C. Preparation of absorbed immune sera: Group-specific antisera were obtained by the immunization of rabbits with human Group II and Group III cells, respectively.[7,8] Only those sera were employed which were found to contain a sufficient quantity of group-specific immune bodies.   An appropriate dilution of

TABLE I.

Agglutination of Chimpanzee and Orang Erythrocytes by Normal Human Serum.

Human sera.	Group.	Blood cells.		
		Chimpanzee E.	Chimpanzee F.	Orang S.
28	I	++	+±	
30	I	+(lysis).	+	
88	I	++		
94	I	++		
16	I			++
96	II	±		
97	II	±		
1	II	0	0	
23	II	Tr.	0	
27	II	+±		
14	II			+(lysis).
17	II			+±
91	III	+++		
93	III	++		
8	III	+++	+++	
J	III	+±	+±	+±
L	IV	Tr.	Lysis.	
G	IV	+		
· 25	IV	+	+	+

Readings after 1 hour at room temperature.

In this and subsequent tables, the following signs are used: 0 = negative; F. tr. = faint trace; Tr. = trace; ±, +, +±, etc.

the sera depending on the strength (we ordinarily used 1/40 dilution) was mixed with 1/2 volume of washed human Group I blood sediment, allowed to stand 1 hour at room temperature and overnight in the refrigerator, and the supernatant fluid was separated by centrifugalization.   Tests were made as described under A above.

D. Grouping with Forssman antiserum: According to the observation of Schiff and Adelsberger[9] certain Forssman heterogenetic antisera (prepared by the im-

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[9] Schiff, F., and Adelsberger, L., *Z. Immunitätsforsch.*, 1924. xl, 335.

munization of rabbits with suspensions for instance of horse kidney) react on human Group II cells. The test with one such serum, which was highly specific in agglutinating human Group II erythrocytes, proved to be useful as a confirmation. (The results are not presented in a table.)

The action of normal human serum on chimpanzee and orang cells is illustrated by the experiments given in Table I; while in Table II, the action of chimpanzee serum on human cells is recorded.

It will be seen that most of the tests indicate that the bloods of Chimpanzees E and F may well correspond to the human Group II, a finding which was established by other methods as well. But the unreliability of grouping done in this way is brought out by the fact that human sera of Group II which are rich in heteroagglutinins can act as strongly on chimpanzee erythrocytes as a serum belonging to

TABLE II.

Agglutination of Human Erythrocytes by Chimpanzee Serum.

		Human blood cells.							
	M	94	95	96	97	98	H	93	L
Group...................................	I	I	II	II	II	II	II	III	IV
Serum Chimpanzee E.............	Tr.	±	0	Tr.	F.tr.	±	Tr.	++	+±

Reading after 1 hour at room temperature.

Group III. Grouping of orang blood by the method is still less certain. If it is to be used at all for grouping chimpanzee blood one must select such sera as contain very strongly active isoagglutinins.

The tests have a bearing on the question of the differentiation of human and anthropoid bloods though we observed an instance of agglutination of chimpanzee cells by chimpanzee serum which was not in accord with the typical blood grouping. But if one take into account all of the tests performed, they are sufficient to establish the species specificity of human and chimpanzee blood.

Tables III, *a* and *b* give examples of experiments by which a satisfactory grouping of the anthropoid bloods was carried out. In the tests on chimpanzee erythrocytes the reactions were either distinctly positive or negative. The same was true of tests with orang and gibbon blood and purified normal agglutinins. In the tests on orang blood with absorbed immune sera the effect could be seen from the

TABLE IIIa.

Agglutination of Orang and Gibbon Erythrocytes by "Agglutinin Solutions" Prepared According to Method B.

Agglutinin solution prepared from	Orang.				Gibbon.	Human.	
	T	U	V	W		II	III
Human Group II serum........	0	+	+	0	0	0	+
" " III "	+	0	0	+	+	+	0

TABLE IIIb.

Agglutination of Chimpanzee Erythrocytes by "Agglutinin Solutions" Prepared According to Method B and Group-Specific Immune Sera According to Method C.

Agglutinin solution prepared from	Chimpanzee.						Human.	
	E	L	M	N	O	P	II	III
Human Group II serum...........	0	0	0	0	0	0	0	+
" " III "	+	+	+	±	+	0	+	0
Immune serum 414 absorbed.......	+++	+++	++±	++	++±	0	+++	0
" " 481 "	0	0	0	0	0	0	0	+±

Readings after 1 hour at room temperature.

TABLE IV.

Absorption of Sheep Hemolysins by Chimpanzee Blood.

Immune serum 422 (anti-human Group II erythrocyte) in 1/100 dilutions absorbed overnight with one-half volume of sediment of each blood. Tests consisted of:

0.5 cc. supernatant fluid.

0.5 cc. fresh guinea pig serum (1/10 dilution).

1 drop 50 per cent suspension of sheep blood.

Readings after 20 minutes in water bath at 37°C.

	Dilutions.				
	100	200	400	800	1600
Control (unabsorbed).........................	C	C	AC	D	0
Absorbed with cells of Chimpanzee E............	0	0	0	0	0
" " " " " F............	0	0	0	0	0
" " human cells Group I.............	C	C	AC	D	0
" " " " " II.............	0	0	0	0	0
" " " " " III............	C	C	AC	D	0

C indicates complete hemolysis; AC, almost complete hemolysis; D, distinct hemolysis; 0, no hemolysis.

difference in the action of the immune serum absorbed with human Group I blood and absorbed with human Group II blood.   These tests showed that with the proper technique chimpanzee and orang bloods can be grouped as exactly as human blood.

No distinct isoagglutinin reactions were observed in the blood of ten *Macacus rhesus* monkeys and four baboons (*Papio papio*).

The experiments with immune serum against the Forssman antigen showed that the similarity existing between chimpanzee and human blood of Group II extends even to the fact that both these cells contain a factor related to the Forssman antigen.

This was also brought out by the following observation: As Schiff and Adelsberger[9] have shown, certain, not all, rabbit immune sera for human Group II red cells contain hemolysins for sheep cells, which can be removed by absorption with human blood of Group II or IV but not by blood of Group I or III.   Table IV shows that this hemolysin is absorbed by chimpanzee blood belonging to Group II, as readily as by human Group II blood.

It is well known that in human blood the isoagglutinins follow the remarkable rule that a blood serum contains isoagglutinins for those isoagglutinogens which are absent from its own cells.   This fact has given rise to discussion on the origin and inheritance of the normal antibodies.   It has been of interest to see whether a similar rule held for the anthropoids.   As we had at our disposal only small quantities of anthropoid serum, the number of experiments which were made to investigate this point is not large.   In all cases, however, in which tests could be made, the isoagglutinins were found to follow the same rule as for human blood (see Tables V and VI).

The faint reactions of chimpanzee serum on orang blood of the same group are probably weak heteroagglutinin reactions.   It is noteworthy that the blood of Chimpanzee F was weakly, but definitely agglutinated by the serum of Chimpanzee E although both belonged to the same group (Group II).   This shows that besides the typical group differences other individual differences exist.   We are not in a position to decide whether or not the two animals concerned belonged to different varieties.

The group reactions which we have observed by the various methods in anthropoid bloods correspond in every respect to the group reactions

TABLE V.

Isoagglutination Reactions with Anthropoid Bloods.
Action of Normal Chimpanzee Serum on Chimpanzee Erythrocytes.

Sera.	Chimpanzee erythrocytes.						
	E	F	H	K	L	M	O
	Group.						
	II	II	II	I	II	II	II
Serum Chimpanzee E..............	0	±	0	0	0	0	0
" " F..............	0	0	0	0			
" " H..............	0	0	0	0			
" " K..............	++	++±	++±	0			

Action of Normal Orang Serum on Orang Erythrocytes.

Sera.	Orang erythrocytes.			
	T	U	V	W
	Group.			
	II	III	III	II
Serum Orang U...............................	+	0	0	++
" " V...............................	+	0	0	+±
" " W...............................	0	+	±	0

Readings after 1 hour at room temperature.

TABLE VI.

Action of Normal Chimpanzee Serum on Orang and Gibbon Erythrocytes.

Sera.	Erythrocytes.					
	Orang.				Gibbon.	Chimpansee E.
	T	U	V	W		
	Group.					
	II	III	III	II	II	II
Chimpanzee E....................	Tr.	+±	+±	Tr.	0	0
" F....................	0	+	+	Tr.	0	0

Readings after 1 hour at room temperature.

in human blood. This is significant in connection with the tests on the bloods of the lower monkeys reported in a communication to follow.[10]

According to one observation of von Dungern and Hirschfeld[5] the chimpanzee blood did not absorb all the isoagglutinins from human serum III. In our experiments, however, which were repeated several times, the human isoagglutinins were practically entirely absorbed by chimpanzee cells.

The typings of the anthropoid bloods according to the foregoing experiments may be summarized as follows: of fourteen chimpanzees three belong to Group I and eleven to Group II as they exist in human blood. Of the two sera and one blood of chimpanzee studied by von Dungern and Hirschfeld,[5] two specimens apparently belong to Group II. The third is considered by these authors to belong also to Group II but it might possibly be assigned to Group I. If we adopt the view of von Dungern and Hirschfeld and add their determinations to ours, a total of seventeen chimpanzees have been grouped, of which three had blood of Group I and fourteen of Group II.

Our results with the orangs are different. Of six individuals two belong to Group II, three to Group III, and one to Group IV. The one gibbon (*Hylobates lar*) examined belonged to Group II.[11]

<center>DISCUSSION.</center>

From the foregoing experiments it may be concluded that the bloods of the anthropoid apes contain groups identical with those of human blood. We were able to assign each blood to one of the four human groups. The occurrence of the agglutinogens of the human type seems to be limited to man and the anthropoids, for the serological factors related to the human isoagglutinogens which are detectable in the blood cells of the lower monkeys and other mammals can be differentiated from the human factors by means of reactions with group-specific immune sera.

The presence of the blood group factors in the apes has evidently a

[10] Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 863.

[11] This finding was incorrectly reported in our preliminary communication (*Science*, 1925, lxi, 492) as Group III.

bearing on the question of the significance and origin of the blood groups in man. The literature on the subject[2,12-16] contains two chief hypotheses, one that the blood groups were present in the different races of primitive man, the other that they arose as relatively recent mutations. If our findings in the anthropoids are taken into consideration, the simplest assumption seems to be that the isoagglutinable factors existed before man and the anthropoids were differentiated from their common ancestor. If this asssumption is not made, one is forced to the conclusion that identical mutations occurred in the evolutionary lines which developed into the gibbon, orang, chimpanzee, and man at some later time.

Paleontological studies have led to the generally accepted conclusion that the existing species of man is only one of a number of human species, the rest of which have become extinct,[17] as for instance the Neanderthal man. If the group factors had evolved before the human stage had been reached they must have been present in these various fossil human species. Either the groups were distributed among them in a manner comparable with the distribution in man of the present day or else in such wise that each species contained only a part of them.

In contemplating the latter case the question presents itself whether or not the crossing of human types more or less pure as regards the groups may have resulted in the puzzling existence in man of a few sharply defined serological qualities and in their distribution. In this respect the findings in anthropoid apes are of interest. In chimpanzees only the isoagglutinogen A (of Group II cells) was encountered. Although the number of individuals examined is not large, and more extensive studies are needed, it is probable that the factor A is either characteristic for these animals or is predominant, and is present in a higher proportion than in any known race of man. A parallel case,

[12] Hirschfeld, L., and Hirschfeld, H., *L'anthropologie*, 1918–19, xxix, 505.

[13] Schiff, F., and Adelsberger, L., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, 172.

[14] Amzel, R., Halber, W., and Hirszfeld, L., *Z. Immunitätsforsch.*, 1925, xlii, 369.

[15] Bernstein, F., *Z. Indukt. Abstammungs.- u. Vererbungsl.*, 1925, xxxvii, 237.

[16] Steffan, *Arch. Rassen- u. Ges.-Biol.*, 1923–24, xv, 137.

[17] See Keith, A., The antiquity of man, London, 2nd edition, 1925.

which can be cited, is that of the North American Indians whose bloods are almost entirely of Groups I and II.

Up to the present no essential difference between group-specific and species-specific agglutinins and agglutinogens has been demonstrated with certainty (see the following paper). One may suppose, therefore, that from the crossing of two species which yield fertile hybrids, an F_2 generation might be obtained in which segregation of the parental characters would result in the appearance of blood groups. For the elucidation of these problems it would be valuable to determine which species of animals exhibit well defined blood groups and whether such species can reasonably be supposed to have had a polyphyletic origin.

SUMMARY.

The bloods of twenty-one anthropoid apes have been examined serologically. It was found possible to assign each to one of the four human blood groups.

The isoagglutinogens of the anthropoids were found to be identical with those of human blood. These very same factors could not be demonstrated in the blood of the lower monkeys.

In the blood of the fourteen chimpanzees examined only the isoagglutinogen A has been found, whereas both A and B were present in the blood of the six orangs.

The significance of these findings for the knowledge of the human blood groups is discussed.

SEROLOGICAL STUDIES ON THE BLOOD OF THE PRIMATES.

III. DISTRIBUTION OF SEROLOGICAL FACTORS RELATED TO HUMAN ISOAGGLUTINOGENS IN THE BLOOD OF LOWER MONKEYS.

BY K. LANDSTEINER, M.D., AND C. PHILIP MILLER, JR., M.D.

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It has been shown that agglutinins exist in the sera of various animals, which react on the isoagglutinogens of human erythrocytes, and conversely that there are substances in animal blood cells resembling the human isoagglutinogens.[1] Von Dungern and Hirschfeld found that treatment of normal human serum with the erythrocytes of certain animals, for example the rabbit and the steer, remove a part of the isoagglutinins. Blood cells of other species fail to do this. The factors in the corpuscles which were responsible for the absorption do not completely resemble those in human blood. This is shown by the fact, for instance, that substances are present in rabbit serum which act upon the human agglutinable factor B,[2] but which, as is to be expected, do not react on rabbit blood.

Related findings on the similarity of the factor A to the Forssman antigen have been made by Schiff and Adelsberger.[3]

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[1] Landsteiner, K., *Wien. klin. Rundschau*, 1902, No. 40. von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526. Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419. Halpern, J., *Z. Immunitätsforsch., Orig.*, 1911, xi, 609.

[2] We designate the isoagglutinable factors of human erythrocytes by the usual symbols: A for that in Group II cells; B for that in Group III cells.

[3] Schiff, F., and Adelsberger, L., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, 172; *Z. Immunitätsforsch.*, 1924, xl, 335. Hesser, S., *Acta med. Scand.*, 1924, suppl. 9. Amzel, R., Halber, W., and Hirszfeld, L., *Z. Immunitätsforsch.*, 1925, xlii, 369.

We have undertaken to search for group factors in the bloods of the lower monkeys for two reasons; first, in order to compare the findings with those obtained with the bloods of the anthropoid apes and already reported;[4] secondly, because we considered it desirable to determine by examination of a number of species of certain families of animals whether the presence of the serological factors in the blood cells of animals bears any general relationship to their position in the systematic zoological classification.

It has been pointed out in preceding papers[4-7] that the relationships thus far established between the zoological classification of animals and their serological qualities differ for proteins on the one hand and the agglutinogens and lysinogens of cells, especially erythrocytes, on the other. The relationship is not as evident in the latter case as in the former, the zoological distribution of the serological factors appearing rather irregular. Yet it does not follow that laws of some kind will not be found.

<div align="center">EXPERIMENTAL.</div>

Methods.—The absorption method was not suitable for our purpose because we often had at disposal only small quantities of blood. This method has the additional disadvantage of measuring only the gross differences between treated and untreated sera, not giving clear-cut results when but a small fraction of the antibody is absorbed. We have, for these reasons, employed the technique used in the experiments on the anthropoid apes; namely, agglutination tests by means of "purified agglutinin solutions," absorbed group-specific immune sera, and a heterogenetic (Forssman) antiserum. These methods are described as Methods B, C, and D in the preceding communication.[4]

In Table I are given some representative tests on monkey bloods. The following list contains the species examined, arranged according to families and genera. The number of individual members of each species tested appears in parentheses whenever that number exceeds unity.

[4] Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 853.

[5] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91.

[6] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xlii, 123.

[7] Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 841.

TABLE I.

Agglutination Tests on Monkey Erythrocytes.*

The agglutinin solutions were prepared from human Group II and Group III sera according to Method B. The immune sera were group-specific, anti-human, erythrocyte sera (Group II and Group III), which had been absorbed with human Group I cells according to Method C. Serum 54 was a heterogenetic (Forssman) antiserum prepared by the immunization of rabbits with horse kidney (Method D).

	Macacus rhesus.	Macacus rhesus.	Mangabey.	Moustached monkey.	Pig-tailed monkey.	Bonnet monkey.	Green monkey.	Vervet monkey.	Sapajou.	Sapajou.	Woolly monkey.	Howler.	Lemur.	Chimpanzee (Group II).	Human (Group II).	Human (Group III).
Agglutinin solution from human Group II serum	0	F. tr.	0	0	0	0	0	0	+	+±	+	+	+	0	0	+
Agglutinin solution from human Group III serum	0	0	0	0	0	0	0	0	0	Tr.	0	0	F. tr.	+	+	0
Absorbed immune serum for Group II	0	0	0	0	0	0	0	0	0	Tr.	0	0	+	+++	++	0
" " " III	0	0	0	0	0	0	0	0	0	Tr.	0	0	0	0	0	+±
" " 54 (heterogenetic)	Tr.	F. tr.	0	0	0	0	±	+	0	Tr.	Tr.	+	++	++	++	±

Readings after 2 hours at room temperature.

* In the tables the following signs are used: 0 = negative; F. tr. = faint trace of agglutination; Tr. = trace; ±, +, + ±, etc.

List of Monkeys.

<div align="right">No.
examined.</div>

I. Old World Monkeys.   Cercopithecidæ.

Baboons—Genus *Papio*.

Guinea baboon.............................*P. papio* or *P. sphinx.*   (3)

Mantled   "   ....*P. hamadryas.*

Species uncertain; either *P. porcarius* or *P. cynocephalus.*   (4)

Guenon Monkeys.   Genus *Cercopithecus*.

Green   monkey...........................*C. sabæus.*   (2)

Vervet   "   ....*C. pygerythrus.*   (3)

Patas   "   ....*C. patas.*

Mona   "   ....*C. mona.*   (3)

Moustached   "   ....*C. cephus.*

Hochuer   "   ....*C. nictitans.*

Mangabeys.   Genus *Cercocebus*.

Sooty mangabey.............................*C. fuliginosus.*   (2)

White-collared mangabey......................*C. collaris.*

Gray-cheeked   "   ....*C. albigena.*

Macaque Monkeys.   Genus *Macacus*.

M. rhesus.................................   (16)

Java monkey.................................*M. cynomolgus.*   (2)

Lion-tailed monkey...........................*M. silenus.*

Bonnet monkey...............................*M. sinicus.*   (2)

Pig-tailed monkey...........................*M. nemestrinus.*

Black macaque...............................*M. maurus.*

II. New World Monkeys.   Platyrrhina.

Family Cebidæ.

Sapajous, or Capuchin Monkeys.   Genus *Cebus*.

Brown sapajou ....*C. fatuellus.*   (2)

Weeper   "   ....*C. capucinus.* }   (5)

White-fronted sapajou......................*C. albifrons.* }

White-throated   "   ....*C. hypoleucus.*

Black-footed *Cebus*.........................

Woolly Monkeys.   Genus *Lagothrix*.

Humboldt's woolly monkey.................*L. lagotrica.*   (3)

Spider Monkeys.   Genus *Ateles*.

Black spider monkey.......................*A. ater.*   (3)

Black-headed spider monkey................*A. cucullatus.*

Howling Monkeys.   Genus *Mycetes*.

Red howler...............................*M. seniculus.*

Squirrel Monkeys.   Genus *Chrysothrix*.

Chrysothrix (species uncertain; probably *sciurea*).

Douroucolis.   Genus *Nyctipithecus*.

Three-banded douroucoli....................*N. trivirgatus.*

Family Hapalidæ.

Marmosets.

Common marmoset........................*Hapale jacchus.*   (3)

Lemurs.
    Family Lemuridæ, of the suborder Lemuroidea.
        True Lemurs.   Genus *Lemur.*
            Mungoose lemur.............................*L. mungos.* (2)
            White-fronted lemur.........................*L. albifrons.*
            Black lemur................................*L. macaco.* (2)
            Ring-tailed lemur............................*L. catta.*
            Red-brown   " ....*L. rufifrons.*
            Spotted lemur...............................

In addition the bloods of two mandrills (*Papio maimon*) and one drill (*Papio leucophæus*) were obtained, but they could not be satisfactorily examined because for unknown reasons spontaneous clumping of the cells occurred in suspensions of the washed cells in saline.

A total of 76 individuals of 36 species has been examined with the following results:

The 46 individuals of the 18 species of Old World monkeys of the family Cercopithecidæ (Catarrhina) gave, in almost all instances, entirely negative reactions with the agglutinin solutions prepared from normal human Group II and Group III serum. The rare faint reactions which occurred may or may not have been due to imperfections in the technique.

In striking contrast to the results with the Cercopithecidæ were those obtained with the New World monkeys (Platyrrhina). All of the 22 individuals of the 12 species examined gave strongly, or at least distinctly, positive reactions[8] with the agglutinin solution Group II. With the absorbed group-specific immune serum they gave either negative or very weak reactions, which latter, in our opinion, are not due to group-specific antibodies, but rather to the heteroagglutinins present in the (rabbit) sera.

In the same manner the bloods of some members of the family of lemurs (Lemuroidea), of which 8 individuals of 6 species were examined, reacted positively with the Group II agglutinin solution.

The blood of 3 or 4 species of lemurs examined reacted positively with Forssman antiserum. The agglutination of 2 of these bloods by Group II immune serum may be connected with this reaction. In one case which was negative with Forss-

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[8] Only a weak reaction was obtained with one blood of douroucoli (*Nyctipithecus trivirgatus*), not contained in the list, the only instance in which blood of a dead animal was used.

man antiserum a moderate agglutination took place with Group III immune serum. The significance of this reaction could not be determined by absorption tests because of lack of material.

For comparison absorption experiments were carried out in some cases. The results were in complete agreement with those obtained with our routine method. An example is given in Table II, which shows that the blood of the two Platyrrhina (sapajous) absorbed almost the whole agglutinin content of the normal human Group II

TABLE II.

Absorption of Normal Human Serum by Monkey Bloods.

First absorption: Normal human serum (Group II) absorbed for 1 hour at room temperature and overnight in the ice box with one-half volume of packed, washed sediment of each monkey blood. Tests made as follows:

0.05 cc. absorbed serum.
0.1 cc. salt solution.
0.1 cc. 2.5 per cent suspension of human Group III erythrocytes.

Second absorption: Supernatant absorbed again for 1 hour at room temperature with one-fourth volume of packed sediment of each monkey blood. Tests made as follows:

0.1 cc. absorbed serum.
0.1 cc. salt solution.
0.1 cc. 2.5 per cent suspension of human Group III erythrocytes.

Human serum Group II.	Erythrocytes used for absorption.				
	Macaque.	Baboon.	Sapajou.	Sapajou.	
Before absorption...................					+±
After 1st absorption.................	+±	+±	F.tr.	F.tr.	
" 2nd "	+±	+±	Tr.	F.tr.	

serum, while the blood of the two Cercopithecidæ (baboon and macaque) failed to diminish the agglutinin reaction.

Titration of the agglutinin solution for Group II against one blood each of the Cercopithecidæ and Platyrrhina (macaque and sapajou) showed that in the case of the former no reaction occurred with 6 drops of the solution, while the smallest quantity which still agglutinated the latter was 1 drop of a 1/8 dilution.

In addition to the tests on monkeys the bloods of other animals have been examined in the same manner as the monkey bloods. Some examples of these tests are given in Table III.

TABLE III.

Agglutination Tests on Erythrocytes of Other Animals.

For methods see the legend to Table I.

	Mouse.	Rabbit.	Rabbit.	Rat.	Guinea pig.	Duck.	Pigeon.	Goose.	Donkey.	Horse.	Pony.	Goat.	Cat.	Dog.	Human II.	Human III.
Agglutinin solution from human Group II serum..........	+	±	±	±	+	o	o	o	+	o	o	o	+	±	o	+
Agglutinin solution from human Group III serum..........	o	Tr.?	+	Tr.	o	o	o	o	o	o	o	o	o	Tr.?	+	o
Absorbed immune serum for Group II....	o	o	o	Tr.	o	+	o	±	o	o	o	o	o	Tr.	+	o
" " " III....	o	o	o	o	o	+	o	Tr.	o	o	o	o	o	o	o	±

Readings after 2 hours at room temperature.

Tests with the agglutinin solution prepared from human Group II serum gave distinctly positive reactions on the bloods of rabbit, guinea pig, rat, mouse, cat, dog, steer, donkey, and pig, and negative reactions on sheep, horse, goat, pigeon, chicken, duck, and goose bloods.   The Group III agglutinin solution gave positive reactions only on the bloods of rabbit and sheep, and negative reactions on those of all the rest.   The occurrence in this series of a considerable number of positive reactions with the Group II agglutinin solution is in accordance with the findings of von Dungern and Hirschfeld already mentioned.   These authors found distinct individual differences by means of absorption experiments, but in a limited number of such tests we found either no difference, or, at the most, only slight ones.

DISCUSSION.

It follows from the results described that, as has been previously shown, the isoagglutinins of normal human serum act upon the red blood cells of a considerable number of animals, and the conclusion may therefore be drawn that serological substances similar to the human isoagglutinogens have a widespread distribution in the animal kingdom.   But it has also been demonstrated by means of immune sera[9] from rabbits that the structures in the human and animal erythrocytes, though similar, are not identical.   For the absorbed group-specific immune sera failed to give any definite reactions with the animal erythrocytes susceptible to the action of normal human isoagglutinins.   In our studies only the blood of the anthropoid apes gave such reactions, a fact attesting to the close relationship with man.[10]

The bloods of ducks and geese reacted with both Group II and Group III immune sera, the former moderately, the latter slightly.   But these reactions of duck blood were found to be due to heteroagglutinins which did not correspond to the group agglutinins, for the reactions persisted after treatment of the sera with human Group II and Group III cells.   No test of the sort was made with goose blood.

The investigation of the bloods of the lower monkeys brought out an unexpected regularity in the distribution of agglutinogens.   Up to the present the zoological distribution of the few agglutinable factors

[9] Sera obtained by the immunization of chickens behaved in a different way. The problem presented by this difference will not be considered here.

[10] It is possible though not probable that the one case mentioned above of a lemur constitutes an exception.

studied by previous workers has, on the whole, appeared to be quite irregular. To be sure, the factor related to B had been found only in mammals, not in birds, but a very small number of species of the latter
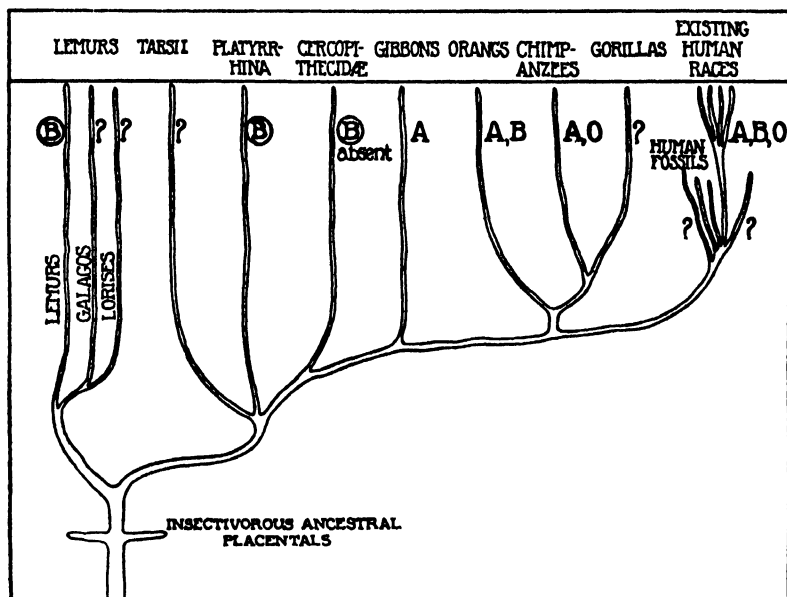
TEXT-FIG. 1.   Adapted from Keith, A., The antiquity of man, London, 2nd edition, 1925, and Sonntag, Ch. F., The morphology and evolution of the apes and man, London, 1924.

A = agglutinogen of human Group II red cells.
B = agglutinogen of human Group III red cells.
Encircled B = agglutinogen similar to, but not identical with, B.
O = blood and serum corresponding to human Group I.
? = not examined.
Of the gibbons only one individual was examined.

have been examined.   Also the Forssman antigen is usually regarded as exemplifying the irregularity of the distribution of receptors.[11]

[11] Possibly a law is indicated by the fact that some very closely related species are similar in that the Forssman antigen is present in their blood (Schmidt, H., Die heterogenetischen Hammelblutantikörper und ihre Antigene, Leipsic, 1924). In this connection it may be mentioned that a positive agglutination was obtained with the blood of three species of lemurs tested with Forssman antiserum prepared by the injection of horse kidney.

In view of these facts it is noteworthy that we have found by our studies in monkeys that whole genera or families appear to be characterized by the presence of certain serological factors (so called receptors) in their erythrocytes. A factor similar to B was found in all of the bloods of the family Platyrrhina examined (genera *Cebus, Lagothrix, Ateles, Mycetes, Chrysothrix, Nyctipithecus*), in the family of the marmosets (Hapalidæ), and also in the family of lemurs (Lemuridæ). This factor was not found in the blood of the Cercopithecidæ, of which the following genera were examined (*Papio, Cercopithecus, Cercocebus,* and *Macacus*). While it is obviously impossible to maintain that exceptions may not be found subsequently the uniformity of our results thus far would seem indicative of a definite rule. A peculiarity of the phenomenon unlike the phenomena of specificity in precipitin reactions on serum proteins is the lack of a gradual transition from one family to another—its sharp discontinuity. This point is evident from Text-fig. 1 in which the findings on the group factors in primates are presented.

The results of our studies should stimulate further investigation in the same general direction since it is possible that like zoological relations exist in the case of other serological factors. The human isoagglutinin lends itself readily to the search for certain agglutinogens. It is possible that with methods resembling ours the zoological distribution of other serological factors can be investigated.

SUMMARY.

Serological studies on the bloods of thirty-six species of lower monkeys have shown that there exists a correspondence between the distribution of a certain hemagglutinogen and the place of the species in the zoological system.

In twelve species of seven genera of Platyrrhina (New World monkeys) and six species of the genus *Lemur* a factor similar to the human isoagglutinogen B was present; in eighteen species of four genera of Cercopithecidæ (Old World monkeys) it was absent, although the latter are more closely related to man than the former.

It would seem from our findings that a genus, perhaps even a family, of animals may be characterized by a special serological factor. The factor found in the lower monkeys is not identical with the one existing in the erythrocytes of the anthropoid apes and man.

COMPARATIVE STUDIES ON THE METABOLISM OF NORMAL AND MALIGNANT CELLS.*

BY JAMES B. MURPHY AND JAMES A. HAWKINS.

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Warburg and his associates have reported a series of studies on the metabolism of malignant tissues as compared to that of normal tissues.[1] Their first observation, that respiration was considerably less in the Flexner-Jobling rat carcinoma than in normal rat tissues, led them to conclude that the Ringer's solution in which the studies were made lacked suitable material for combustion. Later investigation revealed that the addition of glucose inhibited even the small amount of respiration which took place in the tumor tissue suspended in plain Ringer's. The explanation of this was found to be the fact that lactic acid was produced by glycolysis in sufficient amounts to halt the respiratory exchange. On the other hand glucose failed to influence the respiration of normal tissues such as liver and kidney cells and they were found to produce only very minute amounts of lactic acid. This observation led to a quantitative study of the glycolytic activity of malignant cells.

A general analysis of Warburg's results shows that malignant tumor cells produce three to four times more lactic acid per molecule of oxygen consumed than benign tumors, while 3 to 5 day old chick embryo tissue in the absence of oxygen produces lactic acid at almost the same rate as malignant tissue but in the presence of oxygen nor-

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[1] Warburg, O., and Minami, S., *Klin. Woch.*, 1923, ii, 776. Warburg, O., Negelein, E., and Posener, K., *Klin. Woch.*, 1924, iii, 1062. Warburg, O., *Biochem. Z.*, 1923, cxlii, 317. Minami, S., *Biochem. Z.*, 1923, cxlii, 334 Warburg, O., *Biochem. Z.*, 1924, clii, 51. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309.

For a résumé see Warburg, O., *J. Cancer Research*, 1925, ix, 148.

mal respiration takes place with the formation of very minute amounts of lactic acid.   The normal tissues studied in the presence of oxygen respire in the usual manner but are inert in the absence of oxygen. The retina proved to be an exception and the metabolism was of the same nature as that of malignant tumor tissue.

These most important observations seemed to offer the first quantitative method of differentiation between malignant, benign, and normal tissues.   It was our intention in taking up this investigation to determine the relationship between growth rate of tumors and their degree of glycolytic activity.

The methods employed were those used by Warburg, the details of which may be found in his publications.[2]

Method.—Three very thin free-hand sections were made from the tissue to be studied.   One was placed in a Warburg type of cell with 8 cc. of Ringer's solution containing 0.01 mols glucose, 0.025 mols of bicarbonate per liter adjusted to pH 7.6 by bubbling a mixture of carbon dioxide, 5 per cent, and oxygen, 95 per cent, through the solution.   A second piece of tissue was placed in a cell containing 3 cc. of the same solution and the third piece in a cell containing 3 cc. of the same solution plus 0.04 cc. of a 0.7 per cent solution of potassium cyanide.   A fourth cell containing 5 cc. of the same Ringer's solution, without tissue, was used as a control for changes in temperature and barometric pressure.   The cells were attached to Barcroft manometers and after the residual air had been swept out of the manometer and cells by the carbon dioxide-oxygen gas mixture they were sealed. The manometers were placed on a mechanical shaker with the chambers immersed in a constant temperature bath at 37.5 ± 0.01°C., and shaken.   The manometers were left for 15 minutes in the water bath to allow for equilibrium and were then read every 15 minutes. After the experiment was completed the tissues were removed, rinsed in distilled water, placed in weighing bottles, and dried for 2 hours, in an oven at 100°C.   The tissues were then weighed on a micro balance accurate to 0.01 mg.

It is possible to determine the oxygen consumption and carbon dioxide production in c.mm. as follows:

$$k = \frac{v_g \dfrac{273}{T} + v_F\, \alpha}{P_0}$$

v_g = volume c.mm. of gas in cell and manometer.
v_F = " " " Ringer's solution.
α = absorption coefficient of oxygen or carbon dioxide.
T = absolute temperature of thermostat.
P_0 = normal barometric pressure in mm. Brodie's solution 10,000 mm

$$X_{O_2} = h\left(\frac{k_{CO_2} \cdot k_{O_2}}{k_{CO_2} + \gamma\, k_{O_2}}\right) \qquad X_{CO_2} = \gamma\, X_{O_2}$$

$$X_{O_2} = H\left[\frac{K_{CO_2} \cdot K_{O_2}}{K_{CO_2} + \gamma\, K_{O_2}}\right]$$

h = height of liquid in manometer where small letters and capital letters represent the constants for the cells containing 3 cc. and 8 cc. of Ringer's solution respectively.

$$\gamma = \frac{K_{CO_2} \cdot k_{CO_2}}{K_{O_2} \cdot k_{O_2}} \cdot \frac{H\,K_{O_2} - h\,k_{O_2}}{h\,k_{CO_2} - H\,K_{CO_2}}$$

$X_{CO_2}^{N} = k_{CO_2} \times h$, CO_2 produced by glycolysis in cell containing 3 cc. of Ringer's solution plus potassium cyanide.

$$Q_{O_2} = \frac{X_{O_2}}{M \times t}$$ = respiration. C.mm. of O_2 consumed per hour by 1 mg. per dried weight of tissue.

$$Q_{CO_2} = \frac{X_{CO_2}}{M \times t}$$ = total aerobic CO_2 produced per hour by 1 mg. per dried weight of tissue.

$$Q_{CO_2}^{O_2} = Q_{CO_2} + Q_{O_2}$$ = aerobic glycolysis. C.mm. of CO_2 produced by glycolysis per hour by 1 mg. per dried weight of tissue.

$$Q_{CO_2}^{N} = \frac{X_{CO_2}^{N}}{M \times t}$$ = anaerobic glycolysis.

M = weight of dried tissue in mg.
t = time in hours.

Following are some examples of experiments:

Normal Tissue.

		Cell 1.	Cell 2.	Cell 3. (Contains 0.04 cc. of 0.7 per cent KCn to check respiration.)
Gas mixture..................		5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.
Volume, *cc.*..............		$v_F = 8$ $v_g = 5.02$	$v_F = 3$ $v_g = 9.02$	$v_F = 3.0$ $v_g = 10.13$
Constants..................		$K_{O_2} = 0.46$ $K_{CO_2} = 0.89$	$k_{O_2} = 0.80$ $k_{CO_2} = 0.96$	$k_{CO_2} = 1.06$
Rat liver.	Weight of tissue.	2.02 mg.	1.45 mg.	1.93 mg.
	Pressure.	15 min. $H = -4.3$. 30 min. $H = -8.5$.	15 min. $h = -0.3$. 30 min. $h = -0.6$.	15 min. $h = 1.1$. 30 min. $h = 2.9$.
		$\gamma = -1.1$ $Q_{O_2} = -8.9$ $Q_{CO_2}^{O_2} = 1.0$		$Q_{CO_2}^{N} = 3.2$

Embryonic Tissue.

		Cell 1.	Cell 2.	Cell 3. (Contains 0.04 cc. of 0.7 per cent KCn.)
Gas mixture..................		5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.
Volume, *cc.*..................		$v_F = 8.0$ $v_g = 5.02$	$v_F = 3.0$ $v_g = 9.02$	$v_F = 3.0$ $v_g = 10.13$
Constants..................		$K_{O_2} = 0.46$ $K_{CO_2} = 0.89$	$k_{O_2} = 0.80$ $k_{CO_2} = 0.96$	$k_{CO_2} = 1.06$
Skin of rat embryo.	Weight of tissue.	5.25 mg.	5.18 mg.	5.51 mg.
	Pressure.	15 min. $H = -7.1$. 30 min. $H = -14.7$.	15 min. $h = -0.6$. 30 min. $h = -1.6$.	15 min. $h = 9.1$. 30 min. $h = 19.2$.
		$\gamma = 1.1$ $Q_{O_2} = 5.9$ $Q_{CO_2}^{O_2} = 0.65$		$Q_{CO_2}^{N} = 7.4$

Malignant Tissue.

		Cell 1.	Cell 2.	Cell 3. (Contains 0.04 cc. of 0.7 per cent KCn.)
Gas mixture.		5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.
Volume, *cc*..................		$v_F = 8.0$ $v_g = 5.02$	$v_F = 3.0$ $v_g = 9.02$	$v_F = 3.0$ $v_g = 10.13$
Constants...................		$K_{O_2} = 0.46$ $K_{CO_2} = 0.89$	$k_{O_2} = 0.80$ $k_{CO_2} = 0.96$	$k_{CO_2} = 1.06$
Flexner-Jobling tumor.	Weight of tissue.	2.28 mg.	1.71 mg.	1.76 mg.
	Pressure.	15 min. $H =$ 11.2. 30 min. $H =$ 22.4.	15 min. $h =$ 10.4. 30 min. $h =$ 20.9.	15 min. $h =$ 12.5. 30 min. $h =$ 24.2.
		$\gamma = -4.08$ $Q_{O_2} = -8.1$ $Q_{CO_2}^{O_2} = 24.8$		$Q_{CO_2}^N = 29.1$

The tissues studied include the liver, the Flexner-Jobling carcinoma, spleen, embryonic skin and placenta from the rat, spontaneous tumors of mice, and two transplantable sarcomas of the chicken.

Liver.—In general the results with this tissue correspond closely with the figures published by Warburg. There was little variation between the individual livers studied as indicated in Text-fig. 1 and Table I.

Flexner-Jobling Rat Carcinoma.—With this tissue the ratios were likewise fairly constant and corresponded with the published results. These figures may be taken as the standard malignant tumor type of metabolism according to Warburg's classification. (Text-fig. 1 and Table I.)

Spleen.—The spleen gave values slightly different from the liver as may be seen in Table I, but, with one exception, there was little variation between individual spleens. Spleens depleted of the major portion of the lymphoid tissue or stimulated by means of dry heat,[3] showed no measurable difference in their metabolism and the rate was

[3] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.
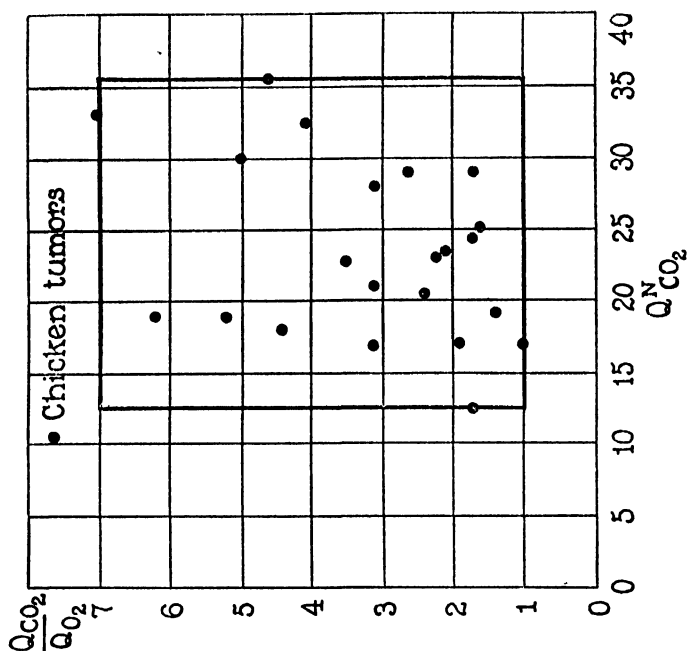
TEXT-FIG. 1.

TEXT-FIG. 2.

TEXT-FIG. 1. The total aerobic carbon dioxide production-respiration ratio are plotted as ordinates and the anaerobic glycolysis as abscissæ. Each point represents an individual observation. The enclosed areas indicate the range of variation for each type of tissue studied.

TEXT-FIG. 2. For explanation see Text-fig. 1.
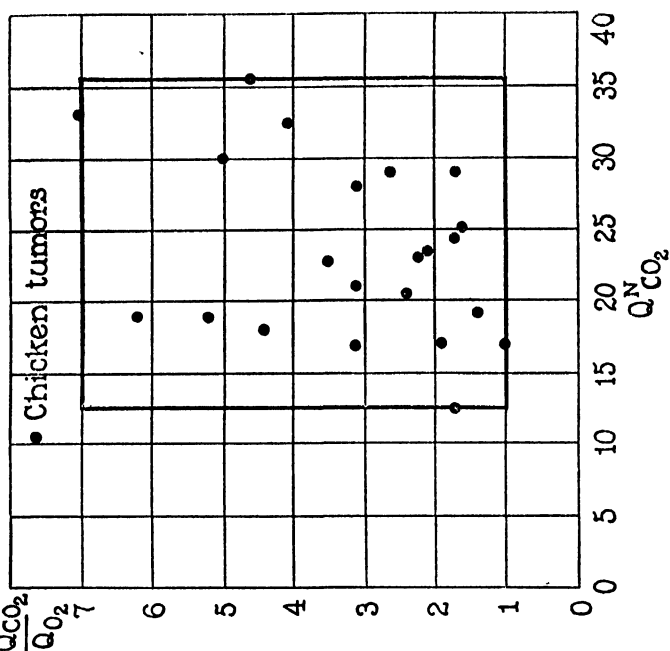
TABLE I.

	No.	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$	$\dfrac{Q_{CO_2}^{O_2}}{Q_{O_2}}$	$\dfrac{Q_{CO_2}^{N} - Q_{CO_2}^{O_2}}{Q_{O_2}}$
Rat liver.	1	−4.97	0.0	1.55	0.0	0.31
	2	−4.97	0.0	2.03	0.0	0.40
	3	−10.6	3.4	0.8	0.3	0.0
	4	−8.9	1.0	3.2	0.11	0.26
	5	−14.2	0.0	2.2	0.0	0.15
	6	−10.1	0.0	1.8	0.17	0.0
	7	−12.5	0.0	3.1	0.0	0.25
	8	−3.8	0.0	0.0	0.0	0.0
	9	−11.8	0.0	3.0	0.0	0.25
	10	−11.6	0.0	3.0	0.0	0.25
Average..................		−9.4	0.44	2.1	0.06	0.19
Flexner-Jobling tumor.	1	−8.6	18.0	26.9	2.0	1.0
	2	−3.3	14.3	17.6	4.3	4.9
	3	−11.7	19.3	27.6	1.5	0.7
	4	−6.6	24.1	24.8	1.6	0.1
	5	−7.6	19.7	30.4	2.6	1.4
	6	−12.2	19.5	30.8	1.6	0.9
	7	−10.4	21.3	26.3	2.0	0.5
	8	−8.4	25.6	29.1	3 0	0.4
	9	−6.9	13.5	47.7	2.0	5.0
	10	−10.5	22.5	28.2	2.1	0.6
	11	−5.3	20.3	28.6	3.9	1.6
Average..................		−8.3	19.9	30.7	2.4	1.5
Rat spleen.	1	−19.3	7.0	5.6	0.3	0.07
	2	−11.4	3.5	9.8	0.3	0.55
	3	−4.0	0.0	6.0	0.0	1.5
	4	−10.9	0.8	9.3	0.1	0.9
	5	−10.0	3.4	6.2	0.3	0.3
	6	−11.1	2.3	9.9	0.2	0.7
	7	−6.7	0.0	5.0	0.0	0.8
	8	−13.1	0.0	5.2	0.0	0.4
	9	−13.6	1.0	11.3	0.1	0.7
	10	−13.5	3.8	9.6	0.3	0.4
	11	−14.7	3.4	9.1	0.2	0.4
	12	−16.9	3.9	10.0	0.2	0.4
	13	−14.3	2.6	11.7	0.2	0.7
	14	−14.8	3.3	5.4	0.2	0.2
	15	−14.6	2.7	10.9	0.2	0.6
	16	−10.0	0.0	8.2	0.0	0.8
Average..................		−11.8	2.3	8.3	0.2	0.6

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TABLE I—*Concluded.*

	No.	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$	$\dfrac{Q_{CO_2}^{O_2}}{Q_{O_2}}$	$\dfrac{Q_{CO_2}^{N}-Q_{CO_2}^{O_2}}{Q_{O_2}}$
Rat placenta.	1	−7.5	11.6	17.4	1.5	0.77
	2	−3.7	9.3	17.8	2.4	2.3
	3	−10.6	12.7	13.4	1.2	0.66
	4	−8.6	9.6	10.8	1.1	0.14
	5	−7.3	11.3	14.4	1.6	0.42
	6	−4.4	6.9	10.2	1.6	0.75
	7	−9.1	11.6	18.6	1.3	0.77
Average..................		−7.3	10.6	14.9	1.5	0.83

Q_{O_2} = respiration. C.mm. of oxygen consumed per hour by 1 mg. per dried weight of tissue.

$Q_{CO_2}^{O_2}$ = aerobic glycolysis. C.mm. of carbon dioxide produced by glycolysis per hour by 1 mg. per dried weight of tissue.

$Q_{CO_2}^{N}$ = anaerobic glycolysis. C.mm. of carbon dioxide produced per hour by 1 mg. per dried weight of tissue when respiration has been checked by potassium cyanide.

$\dfrac{Q_{CO_2}^{O_2}}{Q_{O_2}}$ = ratio of aerobic glycolysis to respiration.

$\dfrac{Q_{CO_2}^{N}-Q_{CO_2}^{O_2}}{Q_{O_2}}$ = Meyerhof quotient.

approximately the same as that of the normal spleen. The one exception was a specimen from a mouse with an old encapsulated abscess in the lower abdominal cavity. This spleen was large and firm, composed almost entirely of enormous germinal centers with only a slight amount of pulp. The type of metabolism was similar to that of a benign tumor. (Text-fig. 1 and Table I.)

Placenta.—The rat placentas investigated were for the most part removed during the last few days of gestation. A few, however, were studied at about mid-term. As there was no difference observed, these are all grouped together in the charts. The type of metabolism was similar to that of frank malignant tissue. (Text-fig. 1 and Table I).

Chicken Tumor.—Chicken Tumor 1, a rapidly growing sarcoma which will cause the death of the inoculated fowl in 3 to 4 weeks,

was of spontaneous origin and has been transplanted through many generations.[4]   This tumor has the unique quality of being transmissible through the agency of a filterable substance separable from the cells.   It is probably the most malignant of the transplantable tumors. The metabolism was similar to that of the Flexner-Jobling rat carcinoma.   (Text-fig. 2 and Table II.)

Chicken Tumor 9, also a transplantable spindle cell sarcoma, but not transmissible by cell-free filtrates, had its origin in a tumor induced by repeated injections of coal tar in the pectoral muscle of a hen.[5] At the time of this investigation Chicken Tumor 9 was failing to grow

TABLE II.

Chicken Tumor 1.

No.	Q_{O_2}	$Q^{O_2}_{CO_2}$	$Q^{N}_{CO_2}$	$\dfrac{Q^{O_2}_{CO_2}}{Q_{O_2}}$	$\dfrac{Q^{N}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}}$	Growth rate.
1	−10.5	17.3	29.2	1.6	1.1	Very rapid progression.
2	−8.9	19.0	21.1	2.1	0.2	" " "
3	−4.5	19.1	26.6	4.2	1.7	" " "
4	−5.3	30.3	33.3	5.7	0.56	" " "
Average....	−7.3	21.4	27.6	3.0	0.9	

For meaning of symbols see Table I.   The tumor used was a rapidly growing chicken sarcoma.

in the majority of fowls inoculated.   With rare exceptions it grew with great slowness requiring 4 months or longer to cause the death of the animal.   In many instances the tumor, after a period of growth, would retrogress rapidly to complete absorption.   Preceding and during this period the sarcoma cells were largely replaced by a small round cell infiltration and an ingrowth of connective tissue.   The anaerobic glycolysis of the tumor was high but the aerobic glycolysis-respiration ratio varied from 0.8, which corresponds to the benign tumor ratio of Warburg, to 4.0 which is the ratio of typical malignant tissue.   Moreover, some of this tissue taken from rapidly retrogres-

[4] Rous, P., *J. Exp. Med.*, 1910, xii, 696; 1911, xiii, 397.   Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52.

[5] Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 807.
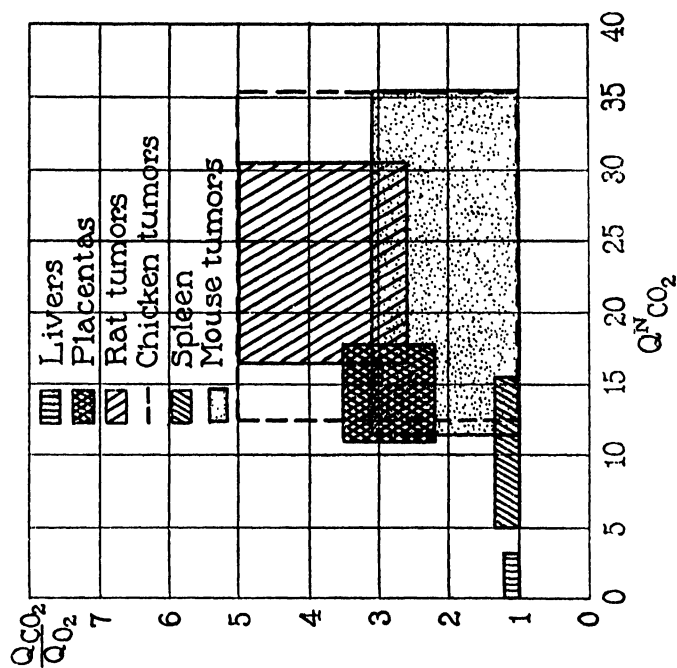
TABLE III.

Chicken Tumor 9.

No.	Q_{O_2}	$Q^{O_2}_{CO_2}$	$Q^{N}_{CO_2}$	$\dfrac{Q^{O_2}_{CO_2}}{Q_{O_2}}$	$\dfrac{Q^{N}_{c} - Q^{O_2}_{CO_2}}{Q_{O_2}}$	Growth rate.	Histological findings.
1	−12.6	9.5	29.1	0.7	1.6	Progressive tumor.	Tumor cells in good condition. No reaction or degeneration.
2	−12.2	8.8	24.9	0.7	1.3	Slow retrogression.	Encapsulated mass, composed mainly of fibrous tissue and lymphoid cells. Some intact tumor cells.
3	−5.7	3.7	12.5	0.7	1.3	"	Very few intact tumor cells. Mostly fibrous tissue and lymphoid cells.
4	−19.0	17.7	17.0	0.9	0.0	"	Practically no tumor cells. Mainly reactive tissue.
5	−16.3	16.3	17.0	1.0	0.0	Progressive, late stage.	Mostly reactive tissue. Small area degenerating tumor cells.
6	−8.4	9.7	23.6	1.1	1.7	Rapid retrogression.	No reaction. Intact tumor cells.
7	−14.7	17.6	23.2	1.2	0.4	Large progressive tumor.	Mainly intact tumor cells. Areas of lymphoid reaction.
8	−12.1	17.2	25.0	1.4	0.6	Young progressive tumor.	Mainly intact tumor cells. Early intense lymphoid reaction.
9	−8.8	12.9	20.6	1.5	1.0	Very slowly progressive.	Intact tumor cells.
10	−8.6	18.4	28.3	2.1	1.1	Young progressive.	" " No reaction.
11	−4.4	9.3	17.0	2.1	1.8	Large	" " " " No reaction.
12	−4.9	12.2	22.8	2.5	2.2	Rapid retrogression.	Mostly intact tumor cells. Early intense lymphoid reaction.
13	−5.7	17.7	32.3	3.0	2.6	Progressive.	
14	−7.0	23.7	18.2	3.4	—	Rapid retrogression.	
15	−4.5	16.1	35.1	3.6	4.2	"	Very few tumor cells. Mass composed mostly of lymphoid cells.
16	−4.2	17.1	30.0	4.0	3.0	Progressive (?).	Composed mainly of tumor cells. Early intense lymphoid reaction.
17	−3.6	18.9	27.0	5.2	2.5	Rapid progression.	Tumor cells intact, very slight reaction.
Average.	−8.8	14.3	23.1	2.0	1.5		

For meaning of symbols see Table I. The growth rate of this chicken tumor was extremely slow and retrogression was frequent and rapid.
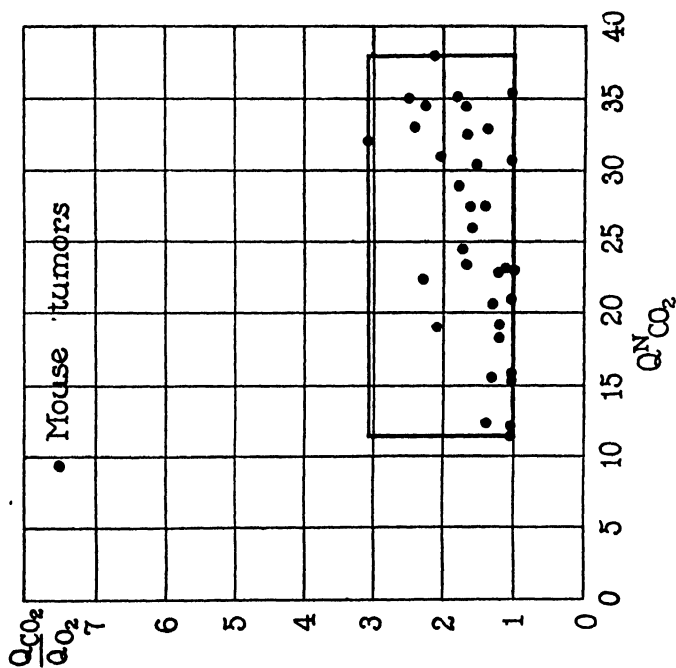
TEXT-FIG. 3. For explanation see Text-fig. 1.
TEXT-FIG. 4. Composite chart showing the range of variation of the tissues studied.

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TABLE IV.

Spontaneous Mouse Tumors.

No.	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$	$\dfrac{Q_{CO_2}^{O_2}}{Q_{O_2}}$	$\dfrac{Q_{CO_2}^{N}-Q_{CO_2}^{O_2}}{Q_{O_2}}$	Diagnosis.	Mitosis.	Growth rate.	Metastasis.	Note.
1	−17.3	0	11.6	0	0.7	Cystic hemorrhagic alveolar carcinoma.	+	?	−	
2	−7.6	0	15.8	0	2.0	Adenocarcinoma.	±	+	−	Developed new tumor.
3	−6.1	0	21.5	0	3.5	Cystic adenocarcinoma.	++	++	−	
4	−12.4	0	15.2	0	1.2	" papillary alveolar carcinoma.	+	?	−	Second tumor grew rapidly.
5	−14.9	0	12.1	0	0.8	Cystic hemorrhagic adeno-carcinoma.	±	+	−	Multiple tumors.
6	−14.6	0	15.6	0	1.1	Adenocarcinoma.	±	+	−	
7A	−17.8	3.9	18.4	0.2	0.3	Cystic hemorrhagic alveolar carcinoma.	++	+++	−	
8A	−17.6	3.7	19.2	0.2	0.9	Adenocarcinoma.	±	++	−	
9	−13.9	2.8	23.0	0.2	1.5	Alveolar carcinoma.	++/++	?	−	Local recurrence.
10	−26.0	9.6	27.3	0.3	0.7	"	++/++	++/+++	−	
11	−17.4	4.9	15.7	0.3	0.6	Cystic adenocarcinoma.	±	+++	+	
12	−10.0	2.5	23.4	0.3	2.1	"	±±	?	−	
7	−8.4	3.5	12.3	0.4	1.1	Alveolar carcinoma.	+++	?	−	Rapid local recurrence.
13	−18.0	7.6	27.6	0.4	1.1	Adenocarcinoma.	±	+++	−	
14	−17.6	6.1	20.7	0.4	0.6	"	±	±	+	
15	−17.9	7.6	27.6	0.4	1.1	"	+++	+	−	Graft grew.
16	−21.0	8.6	35.1	0.4	1.3	"	+++	?	−	
17	−17.7	6.9	33.0	0.4	1.5	"	±	?	−	

No.						Tumor				Local recurrence
18	−16.3	8.3	30.4	0.5	1.3	Cystic alveolar carcinoma.	++	+++	+	Local recurrence.
19	−9.7	6.2	24.6	0.6	1.9	" papillary adeno-carcinoma.	++	++	+	
20B	−15.5	10.2	34.5	0.7	1.9	Adenocarcinoma.	+	+++	+	
21	−17.7	12.7	23.4	0.7	0.6	Cystic hemorrhagic adeno-carcinoma.	+	±	−	
22	−15.6	11.2	26.0	0.7	1.0	Alveolar carcinoma.	+	++	−	"
23	−8.3	6.1	32.5	0.7	3.2	Cystic adenocarcinoma.	±	+++	−	
20	−20.0	16.0	35.2	0.8	1.0	Adenocarcinoma.	+	?	−	"
24	−12.4	10.2	29.0	0.8	1.3	Cystic hemorrhagic adeno-carcinoma.	±	+++	+	
25	−14.8	14.3	36.2	1.0	1.5	Papillary hemorrhagic adenocarcinoma.	+	++	+	
10B	−10.7	11.2	38.0	1.0	2.5	Alveolar carcinoma.	++	+++	−	"
26	−17.5	17.1	30.6	1.0	0.8	" "	+	?	−	
27	−4.4	4.8	23.5	1.1	4.2	Sarcoma (?).		++	−	"
10A	−11.2	12.4	31.1	1.1	1.7	Alveolar carcinoma.	++	+++	−	"
22A	−7.6	8.2	18.7	1.1	1.4	" "	+	++	−	
28	−8.0	10.6	34.9	1.3	3.0	Adenocarcinoma.	++	+++	−	"
29	−11.6	15.6	22.6	1.3	0.6	Alveolar carcinoma.	±	±	−	
27A	−14.1	20.3	33.1	1.4	0.9	Giant cell sarcoma.		+++	−	"
30	−3.2	7.2	32.0	2.2	7.7	Spindle cell sarcoma	±	+++		"
Average....	13.7	7.5	24.7	0.6	1.6					

For mitosis in the tumors ± indicates only an occasional mitotic figure, +++ indicates large numbers, and + and ++ are grades between these extremes. The growth rate where recorded is based on observation for 4 weeks or longer. ± indicates very slight growth; +++ indicates tumors which doubled in size in 4 weeks, and the others are intermediate grades. A, following a tumor number, indicates that the observations were made on an autograft of the original tumor and B, observations on local recurrence following surgical removal.

sing tumors, showing practically no intact tumor cells, the mass being composed almost entirely of reactive tissue, gave as high an aerobic glycolysis-respiration ratio as other tissues composed entirely of malignant cells.   Classification according to this ratio gives no indication as to the state of the tumor.   Compared with Chicken Tumor 1, which has an average malignancy tremendously greater than the most malignant of the chicken tumors, No. 9, during this period of investigation, the average of ratios was not far apart.   Even some of the retrogressing No. 9's gave a ratio higher than the average for Chicken Tumor 1.   It should be noted in this connection that the latter tumor contains large quantities of mucoid material which means that it has considerably fewer cells per unit of tissue than the progressive Chicken Tumor 9.   (Text-fig. 2, Table III.)

Spontaneous Mouse Tumors.—This group of neoplasms has been derived from a special stock of mice which for generations has given a high tumor instance.   Two of the tumors proved to be sarcomas but all of the others were either adenocarcinomas or alveolar carcinomas or a mixture of these two types.   The diagnosis has been recorded in Table IV according to the dominating type.   In a few instances the metabolism rate was studied without determining the rate of growth of the tumor but as a rule accurate measurements were made on the size of these growths for at least a month before the tumors were removed for study.   In some animals the tumors were removed at operation for metabolism study and then a small bit was replaced in the original animal and later a second test was made on the new tumor resulting from this graft, or on the local recurrence at the site of the operation.

The anaerobic glycolysis of these tumors was always high, but the aerobic glycolysis-respiration ratio varied from 0, corresponding to the metabolism of embryonic tissue, to 2.2 which is within the ratios given by the Flexner-Jobling rat tumor.   The majority of these tumors, however, would fall in the group of benign tumors classified on their metabolism according to Warburg's types.   Only two gave a ratio as high as the lower ratios for the Flexner-Jobling tumor and these were both sarcomas.   (Text-fig. 3 and Table IV.)

The aerobic glycolysis-respiration ratio seemed to bear no relationship to the growth rate.   There are more of the rapidly growing tumors

with a high ratio than with a low one, but one of the slowest growing tumors gave one of the highest ratios while several rapidly growing tumors gave very low ratios. In not a single instance was there sufficient stroma or reaction to account for the type of metabolism.

From a biological point of view these tumors unquestionably belong to the group of frankly malignant neoplasms. They grow more or less rapidly, almost invariably resulting in the death of the animal. Spontaneous healing is extremely rare. They invade surrounding tissues, metastasize to the internal organs, recur frequently after careful surgical removal, and autografts almost invariably grow. Recent investigation has shown that a majority, if not all, of such tumors are transplantable if the genetic make-up of the new host is similar to that of the tumor. In other words the spontaneous mouse tumors are capable of continuous growth and in all other respects answer the requirements necessary to group them without question among the malignant neoplasms.

Miscellaneous Tissues.—Skin from rat embryos in the latter stages of gestation, embryonic membranes, and the wall of a pregnant uterus were all found to give the same type of metabolism as the chick embryos.

DISCUSSION.

Briefly put, Warburg divides tissues into four types on the basis of his metabolism studies, namely normal resting tissue with a slight anaerobic glycolysis and a high respiratory rate; embryonic tissue with a high respiratory rate and a high anaerobic but a low aerobic glycolysis; malignant tumor tissue with a low respiration and a high aerobic and anaerobic glycolysis; benign tumor tissue with the same type of metabolism as the malignant tissue but less active glycolytic function.

In our experiments, working with the same types of tissues as those used by Warburg, the results were essentially the same as his and the differences between the groups were quite clear-cut. However, when the observation was extended to other tissues the groups became less definite. For example, rat spleen, embryonic skin, and the wall of a pregnant uterus had the typical embryonic type of metabolism. Rat placenta grouped according to this scheme fell well within the malignant tumor group. The transplantable Chicken Tumor 1

grouped itself with the transplantable rat carcinoma but Chicken Tumor 9 showed considerable variation. The latter tumor grouped on the basis of the aerobic glycolysis-respiration ratio, in the majority of instances, had about the same range as Chicken Tumor 1 but a rapidly retrogressing tumor composed almost entirely of reactive tissue was frequently as active as the progressive tumors made up entirely of intact tumor cells.

The results with the spontaneous tumors of mice were even more varied. Grouped on the basis of their type of metabolism some of them behaved as embryonic tissue but the majority fell in the group of benign tumors and only a very small number could be classed as malignant. Histologically all of these tumors are typically malignant. Classified on the basis of their biological behavior they are in all essentials similar to the malignant disease as it occurs in man.

The glycolytic activity seems to bear no relationship to growth rate. The very slow growing or retrogressing chicken tumor frequently gave as high a ratio as the rapidly growing tumor. The rapidly progressing spontaneous mouse tumors often gave a low ratio while the slower ones in some instances gave a relatively high ratio.

From these results it would seem that a classification of tissues on the basis of the type of metabolism does not correspond to the biological groupings (Text-fig. 3). These findings in no way detract from the fundamental importance of Warburg's studies for he has opened up a new field of tumor research which promises great possibilities for future investigation.

THE PRODUCTION OF PERSISTENT ALOPECIA IN RABBITS BY X-RAYS OF VARIOUS DEGREES OF HARDNESS.

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These studies are a continuation of work reported recently[1] on the minimum dose of soft x-ray required to produce persistent alopecia of the abdominal skin of white rabbits. For the purposes of that work rays produced at 30 kilovolts, peak, were employed, and the critical dose was found to correspond to the production in air of 2.04×10^{16} pairs of ions per gm. The rabbits were found to react accurately; although eighteen of the exposures fell within 2 per cent of the critical value, half on either side, no single case of inconsistent reaction occurred.

The present paper deals with similar experiments on the effect of somewhat harder rays. The various factors which have to do with the quality and intensity of the radiation are given in Table I. Column 1 shows the peak kilovoltage at which the rays were produced; Column 2, the current in milliamperes; Column 3 the thickness of the aluminium filter in mm.; and Column 4, the target distance in cm.

Apparatus.

The same x-ray plant has been u ed in this work as in the previous work. The plant is known to operate with a high degree of consistency. The experiments were made at various tim s over a period of about 1 year, during which time four different tubes were used, and minor adjustments of the electrical equipment were made. As a means of following the tube efficiency and the effect of adjustment, the rate of ionization of air by the rays was measured from time to time. The apparatus used to make these measurements, together with studies of its trustworthiness, has been discussed.[2] The measurements were made with the ioniza-

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[1] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

[2] Clark, H., *Am. J. Roentgenol. and Radium Therap.*, 1924, xi, 445.

tion chamber in place of the exposed portion of the animal. A necessary correction for the effect of scattered radiation has been included in all values used in this paper. It varies from 2 per cent at 40 kilovolts, to 4 per cent at 70 kilovolts. The method of determining it has been described.[1]

Preparation and Treatment of the Animals.

Only normal healthy white rabbits weighing between 1500 and 2100 gm. were used. The method of preparing and exposing them to the radiation has been described in detail.[1] Before exposure, the abdomen was shaved and covered with a screen of lead-filled rubber in which there are seven apertures, six round, 1 cm. in diameter, and one elongated for the purpose of identification. During exposure, the animal was shielded carefully from the heat of the tube and kept cool by a current of air at room temperature.

TABLE I.

1	2	3	4
40	15	0.5	27
50	12	1.0	27
70	8	1.5	36

RESULTS.

In order to avoid the possibility of bias in making measurements and diagnoses, the work has been divided into two parts from the beginning. One of us (Sturm) has attended to the preparation and exposure of the animals, and to the observation of them thereafter, while the dosage measurements were made by the other (Clark). The two sets of records were not compared until each had been put into final form.

Fig. 1 is the record of the alopecia reactions of the animals. It comprises all of the experiments made at 40 and 50 kilovolts except a few preliminary ones in which the dose given proved to be too small to yield results of interest. The results at 70 kilovolts are not shown since in no case was the exposure sufficient to produce persistent alopecia. Each vertical column of circles gives the record of one rabbit, the seven circles corresponding to the seven skin areas treated. Each circle is so plotted on the vertical scale as to show the corresponding length of exposure in minutes. White circles represent temporary alopecia, the figures within showing the time interval after exposure, in weeks, before the regrowth of hair was observed. Black circles repre-

sent alopecia persistent for at least 10 weeks. In a few cases, a very slight regrowth was first observed at 9 weeks. In no case, however, was a similar observation made at 10 weeks, although most of the animals were kept under observation for some time thereafter. Reference to Fig. 1 shows that with one exception,—in which the regrowth was observed at 7 weeks,—all of the lowest black circles of each group lie on the same horizontal line, although the figures in the highest white circles are variable; five of the six 9's in the figure correspond to doses within 3 per cent of the critical dose as calculated below. It is assumed, therefore, that observations made at 10 weeks or thereafter constitute a fair criterion of persistency. Nine groups of animals are represented in Fig. 1—six at 40 kilovolts and three at 50 kilovolts. The four animals of each group were exposed on the same day to rays from the same x-ray tube.

Table II contains a summary of the ionization measurements together with all of the data from Fig. 1 which are of interest for the purpose of determining the critical dose. In addition to the work at 40 and 50 kilovolts, Table II contains the measurements and data relative to the longest exposures at 70 kilovolts. Column 1 contains the identification numbers of the various groups of animals for purposes of reference to Fig. 1. Columns 2 and 3 show the degree of hardness of the rays (kilovolts), and the number of the tube used, respectively. Column 4 contains the summary of the ionizing powers of the rays when the various tubes were used; the figures given are the numbers of pairs of ions (divided by 10^{14}) produced per gm. of air per minute at the surface of the abdomen of the animal. The critical exposure time for each group of animals dealt with in Fig. 1, lies somewhere between the times which correspond to the highest white circles and the lowest black circles; Columns 5, 6, and 7 of Table II have to do with these highest white circles, and Columns 8, 9, and 10, with the lowest black ones. Of these, Columns 5 and 8 contain the number of circles considered; Nos. 6 and 9 contain the exposure times taken from Fig. 1; and Nos. 7 and 10 contain the products (divided by 10^{15}) obtained by multiplying the exposure times of Columns 6 and 9 respectively by the ionization rates of Column 4.

We may make use of Columns 7 and 10 to estimate the critical dose for each kind of ray, in ionization terms. The critical dose must not

FIG. 1.

be less than any of the values given in Column 7 nor greater than any of the values in Column 10.   At 40 kilovolts no such value appears, either because the measurements are inaccurate, or because the animals do not react consistently, or because the output of x-ray is not constant.   The discrepancy is not great, however; if we may assume that the value given in Column 7 for one animal (*), is too high by 2/3 of 1 per cent, the table becomes consistent for the critical value 3.68 $\times$ 10^{15}.   This value is then defined by 19 black circles, and 9 white circles, all of which lie within 2.5 per cent of it.   Since the crit-

TABLE II.

1	2	3	4	5	6	7	8	9	10
1	40	1	1.19	4	28	3.33	4	31	3.69
2	40	2	1.10	4	32	3.52	4	34	3.74
3	40	2	1.10	1*	34	3.74	3	34	3.74
4	40	3	1.05	4	30	3.15	4	35	3.68
5	40	4	1.20	4	30	3.60	4	31	3.72
6	40	4	1.20	4	30	3.60	4	33	3.96
7	50	1	0.90	4	50	4.50	4	55	4.95
8	50	4	0.955	4	45	4.30	4	50	4.78
9	50	4	0.955	4	46	4.39	4	48	4.58
	70	1	0.49	4	80	3.92			
	70	3	0.51	4	100	5.10			

ical value, as written, implies a higher degree of accuracy than can be claimed for the measurements, it may be taken as 3.7 $\times$ 10^{15} pairs of ions per gm.   At 50 kilovolts, the table is consistent; the critical dose may be taken as 4.5 $\times$10^{15}.   At 70 kilovolts, the critical dose is at least greater than 5.10 $\times$ 10^{15}.

To sum up the foregoing, the critical dose is 3.7 $\times$10^{15} pairs of ions per gm. at 40 kilovolts, 4.5 $\times$10^{15} at 50 kilovolts, and over 5.10 $\times$ 10^{15} at 70 kilovolts.

DISCUSSION.

These experiments, taken with the earlier ones,[1] show that, as regards the alopecia reaction, rabbits respond, under constant conditions of treatment, with little or no individual variation.   They show also that when rays of different degrees of hardness are considered, the

critical dose, expressed in terms of ionization, is not constant; at 30, 40, 50, and 70 kilovolts the critical doses are 2.04, 3.7, 4.5, and over 5.1 respectively (all multiplied by 10^{15} pairs of ions per gm.).   It is of interest to note also that the alopecia, erythema, and pigmentation reactions do not run parallel to each other.   At 30 kilovolts, the critical alopecia dose produced little erythema—none in most cases.   At higher voltages, it produced progressively more pronounced erythema. At 30, 40, and 50 kilovolts, it produced no pigmentation, but at 70 kilovolts, one-half of the animals receiving doses above 3.05×10^{15}, which is, of course, far below the critical dose, became pigmented.

There is no evident explanation of these results.   The dosage values given refer to the surface of the animal.   Hard rays produce more ionization within the body of the animal than soft rays, for the same surface ionization; and to produce the same surface dosage, the exposures are necessarily longer.   It seems quite clear that x-rays produce primary changes in matter only through the medium of ionization produced by secondary beta rays, and that the amount of ionization produced is proportional to the energy absorbed irrespective of the hardness of the ray.   It seems clear also that the relation between the ionization of the skin tissues and that of air is within reasonable limits .independent of the quality of the ray.   The complex effects of x-rays on animals probably result from simple primary reactions.

SUMMARY.

1. The minimum dose of x-ray, of each of three degrees of hardness, required to produce persistent alopecia of the abdominal skin of normal white rabbits has been studied.   40 kilovolt rays filtered by 0.5 mm., 50 kilovolt rays filtered by 1 mm., and 70 kilovolt rays filtered by 1.5 mm. of aluminium were employed.   The 70 kilovolt work is unfinished.

2. Under constant conditions of treatment, the animals were found to react with a high degree of consistency.

3. The values of the critical minimum doses expressed in terms of air ionization (pair of ions per gm. of air divided by 10^{15}), for rays produced at 30, 40, 50, and 70 kilovolt peak, are 2.04, 3.7, 4.5, and over 5.1 respectively.   The 30 kilovolt value is taken from a previous paper.[1]

4. It is shown that the alopecia reaction does not run parallel to the erythema and pigmentation reactions.

THE EFFECT OF ETHYL URETHANE ANESTHESIA ON THE ACID-BASE EQUILIBRIUM AND CELL CONTENTS OF THE BLOOD.*

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Ethyl urethane has been frequently employed when a prolonged mild anesthesia is desired and it has been found particularly useful for keeping animals quiet during long exposures to x-ray. Our intention had been to use it for this purpose but since the experiments were designed to determine the action of x-ray on the circulating leucocytes and the acid-base equilibrium of the blood it was deemed advisable first to test the influence of the anesthetic itself.

Technique.—The chemical methods used were those devised for determining the acid-base equilibrium of small animals. The carbon dioxide content of the whole blood was determined by the Van Slyke method[1] and the pH by Cullen's method as modified by Hawkins.[2] The relative pH values were corrected and a constant subtracted to obtain absolute pH values at 38°C.[3] During the experiments all the animals were kept under uniform conditions both as to diet and to temperature. 12 hours prior to the observations all food scraps were removed from the cages, and during manipulation the animals were handled carefully to avoid exciting them.

Experiment 1.—20 rabbits were bled from the heart and the CO_2 content and pH of the blood were determined. 16 of the 20 were then given intraperitoneally 0.2 cc., per 100 gm. of animal weight, of a 50 per cent solution of ethyl urethane in sterile physiological saline. This amount was sufficient to produce anesthesia in from 30 to 45 minutes, which lasted from 6 to 7 hours. CO_2 and pH determinations were made at 1 and 3 hours after the injection and 1, 2, and 3 days thereafter. The results are given in Text-fig. 1 and Table I.

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[1] Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229.

[2] Hawkins, J. A., *J. Biol. Chem.*, 1923, lvii, 493.

[3] The constants were Rabbit Blood 17, Rat Blood 14.

The pH and CO_2 content of the whole blood of the animals injected with urethane were found to be markedly increased at 1 hour and remained at the high level for 24 hours. The normal level was not reached till 72 hours afterwards. The control animals bled at the same intervals had values within normal limits throughout the experiment.

TABLE I.

Effect of Urethane on the pH and CO_2 Content of the Whole Blood of Rabbits.

No.	0 hr.		1 hr.		3 hrs.		24 hrs.		48 hrs.		72 hrs.	
	CO_2	pH	CO_2	pH	CO_2	pH	CO_2	pH	CO_2	pH	CO_2	pH
1	35.2	7.30	48.5	7.52			42.0	7.52	39.5	7.53	44.4	7.46
2	29.7	7.19	52.2	7.52			55.2	7.49	28.7	7.27	36.2	7.38
3	39.6	7.39	64.2	7.48			56.8	7.59	38.5	7.45	31.6	7.31
4	34.2	7.34	44.2	7.45			58.8	7.57	35.7	7.41	28.4	7.28
5	44.2	7.43	59.7	7.58			74.8	7.58	43.6	7.52		
6	25.4	7.28	51.1	7.47			58.5	7.50				
7	40.5	7.45	48.6	7.50	39.3	7.62	50.2	7.58			30.2	7.32
8	49.7	7.49	59.1	7.57	71.8	7.62	59.5	7.56			40.3	7.42
9	48.6	7.55	62.8	7.61	61.8	7.59	61.0	7.56				
10	31.8	7.49	39.7	7.59	43.3	7.53	42.2	7.57	47.9	7.57		
11	39.5	7.49	55.5	7.55	44.6	7.53	45.9	7.51	44.9	7.52		
12	32.9	7.48			50.7	7.54			40.6	7.55		
13	39.1	7.46			63.1	7.58	46.2	7.55			42.0	7.51
14	24.8	7.31			36.6	7.51	39.1	7.39				
15	46.9	7.43			54.0	7.55						
16	30.3	7.35			40.7	7.52						
Average...	37.0	7.40	53.2	7.53	50.6	7.56	53.1	7.54	39.9	7.48	36.0	7.37
Controls.												
1	42.4	7.49	38.9	7.50	38.9	7.49	38.4	7.47				
2	41.0	7.40	39.6	7.42	33.5	7.42	42.4	7.42				
3	43.3	7.42	45.6	7.44					37.7	7.44		
4	35.6	7.39	35.4	7.39	34.0	7.38			37.4	7.37		
Average...	40.6	7.42	39.9	7.44	35.4	7.43	40.4	7.45	37.5	7.40		

CO_2 content expressed in volumes per cent.

Experiment 2.—As control the CO_2 content and pH of the whole blood of 10 rats were determined. The specimens were obtained by decapitating the animals and allowing the blood to drip under oil into a small glass cone. The determinations were made by the methods already described. 30 rats were now given intraperitoneally 1 cc., for every 100 gm. weight, of a 10 per cent solution of ethyl urethane
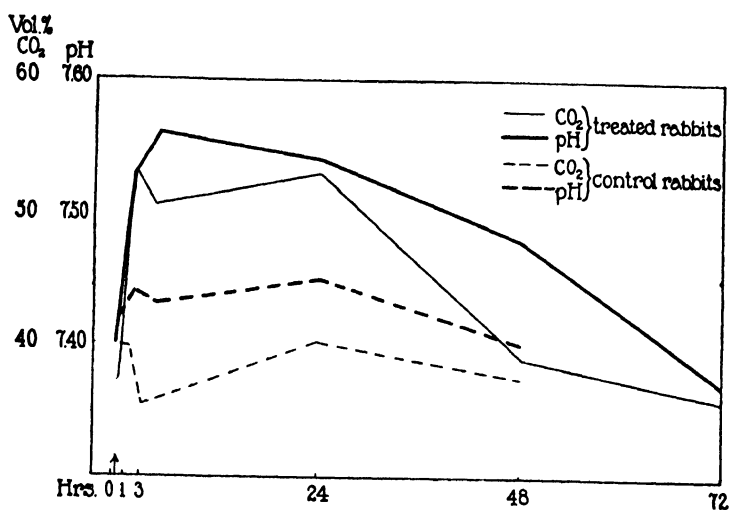
TEXT-FIG. 1.
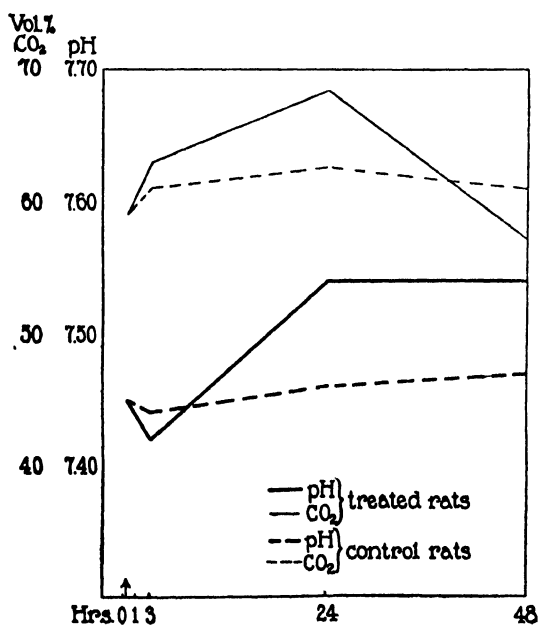
TEXT-FIG. 2.

in sterile physiological saline. This amount was equivalent to that given to the rabbits and produced an anesthesia in 20 to 30 minutes lasting from 6 to 7 hours. At 3, 24, and 48 hours, respectively, after the injection, 10 treated and 5 normal animals were killed and CO_2 and pH determinations made on their blood. The results are shown in Text-fig. 2 and Table II.

TABLE II.

Effect of Urethane on the pH and CO_2 Content of Rat Blood.

0 hr.		3 hrs.		24 hrs.		48 hrs.	
pH	CO₂	pH	CO₂	pH	CO₂	pH	CO₂
7.49	53.4	7.36	65.8	7.53	73.0	7.42	56.4
7.42	62.1	7.41	55.6	7.52	75.6	7.47	60.4
7.41	61.3	7.38	60.0	7.53	70.4	7.49	58.5
7.44	58.4	7.40	69.3	7.56	76.2	7.49	62.0
7.40	56.7	7.43	63.8	7.51	74.3	7.49	54.2
7.43	65.7	7.41	62.7	7.51	57.9	7.56	54.9
7.51	65.4	7.44	61.0	7.52	59.2	7.55	60.4
7.46	57.3	7.53	58.2	7.51	64.4	7.70	56.2
7.47	58.9	7.36	66.2	7.55	58.3	7.63	61.7
7.47	61.8	7.43	58.8	7.56	58.5	7.64	44.5
Average...7.45	59.1	7.42	62.2	7.53	66.8	7.54	56.9
Controls.		7.41	62.7	7.38	63.8	7.57	59.7
		7.44	61.0	7.47	61.9	7.44	66.0
		7.53	58.2	7.48	63.3	7.51	61.0
		7.36	66.2	7.50	63.7	7.40	56.7
		7.43	58.8	7.46	60.7	7.42	62.1
Average...................		7.44	61.4	7.46	62.7	7.47	61.1

CO_2 content expressed in volumes per cent.

The CO_2 content of the whole blood of the animals injected with urethane was found to be increased at 3 hours and reached a maximum at 24 hours after injection. The pH was normal at 3 hours but had increased to a high level at 24 hours and remained at this level at 48 hours. The CO_2 content and pH of the whole blood of the control animals remained within the normal limits throughout the experiment.

These experiments show that a definite alkali excess exists in the blood of both rats and rabbits after injections of ethyl urethane. This is in accordance with the results of Cushny and Lieb[4] who have shown

[4] Cushny, A. R., and Lieb, C. C., *J. Pharmacol. and Exp. Therap.*, 1915, vi, 451.

that in rabbits under deep urethane anesthesia the reaction of the respiratory center to the blood gases is so altered that an increase in CO_2 or a decrease in O_2 has less accelerating action than is the case normally, while CO_2 accumulates in the blood since the rate of breathing is not accelerated to remove the excess CO_2. The CO_2 is retained in the course of the adjustment to restore the alkaline reaction to normal.[5]

The Action of Urethane Anesthesia on the Circulating Leucocytes.— In the course of a study of the action of x-ray on the blood, it was observed that destructive doses of this agent bring about a condition of uncompensated alkalosis.[6] When the same degree of alkalosis was induced by the injection of sodium bicarbonate it was accompanied by the same changes in the circulating white cells which characterized the x-ray effect. As an extension of this observation, we have studied the effect of the alkalosis produced by the injection of urethane on the circulating white cells of the blood.

Experiment 3.—Blood counts were made on 15 normal rabbits and 10 of these were then given a dose of urethane in the same amounts as in the previous experiments. Counts were made on all the animals at 1 and 3 hours after the injections and repeated at daily intervals for a week. The results are given in Text-fig. 3 and Table III.

The average of the counts on the ten treated animals showed a decrease of about 20 per cent in the lymphocytes during the 1st hour, a still greater decrease at the end of the 3rd hour, and a maximum decrease of 60 per cent at the end of the 48 hour period. Thereafter the count slowly returned to the normal level, reaching it by the end of 1 week. On the other hand, the polymorphonuclear leucocytes . increased rapidly after the urethane injection, reaching a maximum of 200 per cent above normal within 24 hours and thereafter slowly returning to normal.

Experiment 4.—Blood counts were made on 39 rats on 2 consecutive days, twenty-six of these being given urethane in the same amount as in Experiment 2. Counts were made on all the animals at 3 hours and at 1, 2, and 6 days after the injection of the urethane. The results are given in Table IV and Text-fig. 4.

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[5] Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 153.

[6] Hussey, R. G., *J. Gen. Physiol.*, 1922, iv, 511.

TABLE III.

Effect of Urethane on the Leucocytes of the Blood of Rabbits.

No.	0 hr. L*	0 hr. P.†	1 hr. L	1 hr. P	3 hr. L	3 hr. P	24 hr. L	24 hr. P	48 hr. L	48 hr. P	96 hr. L	96 hr. P	168 hr. L	168 hr. P
1	2,000	3,100	2,500	4,400	1,600	4,800	2,200	4,900	1,200	3,800	2,700	7,200	3,500	4,600
2	8,100	4,500	5,900	4,500	4,600	4,100	3,000	1,400	2,200	2,500	4,600	2,800	8,700	6,100
3	4,700	4,300	4,300	5,200	3,700	5,500	3,500	4,800	2,600	3,700	2,300	6,500	3,700	5,100
4	6,100	2,400	4,800	3,700	4,000	3,500	3,600	5,900	2,100	3,500	5,300	2,800	3,900	4,200
5	3,000	5,500	2,900	6,400	2,600	7,800	2,400	10,000	3,600	4,900				
6	5,800	3,400	2,400	2,800	4,000	7,600	9,300	10,700	4,800	6,100				
7	5,200	4,000	3,800	5,800	5,100	6,100	1,300	11,100	4,800	8,500				
8	7,200	3,400	5,200	4,100	3,900	5,800	6,800	5,500	3,400	7,500				
9	7,300	2,900	9,000	7,000	8,900	9,400	7,300	5,300	4,500	4,600				
10	5,700	5,000	3,300	5,900	4,900	5,100	6,800	6,200	3,800	6,700				
Average..	5,500	3,900	4,400	5,000	4,200	6,000	4,600	6,600	3,300	5,200	3,800	4,800	5,000	5,000
Controls.														
1	6,600	3,200			6,200	5,000			6,500	3,800			6,800	2,900
2	7,000	2,500			6,800	3,600			7,100	2,800			7,600	3,500
3	4,200	4,100			4,000	5,100			4,300	3,600	4,500	4,300	4,500	3,000
4	5,700	2,600	5,000	3,600	4,800	3,600	6,500	3,800	5,700	3,000	4,900	2,100	4,800	4,000
5	5,800	4,500	5,800	4,300					6,600	3,400	5,900	5,100	5,000	5,100
Average..	5,900	3,400	5,400	3,900	5,400	4,300	6,500	3,800	6,000	3,300	5,100	3,800	5,700	3,800

* Lymphocytes.

† Polymorphonuclear leucocytes.
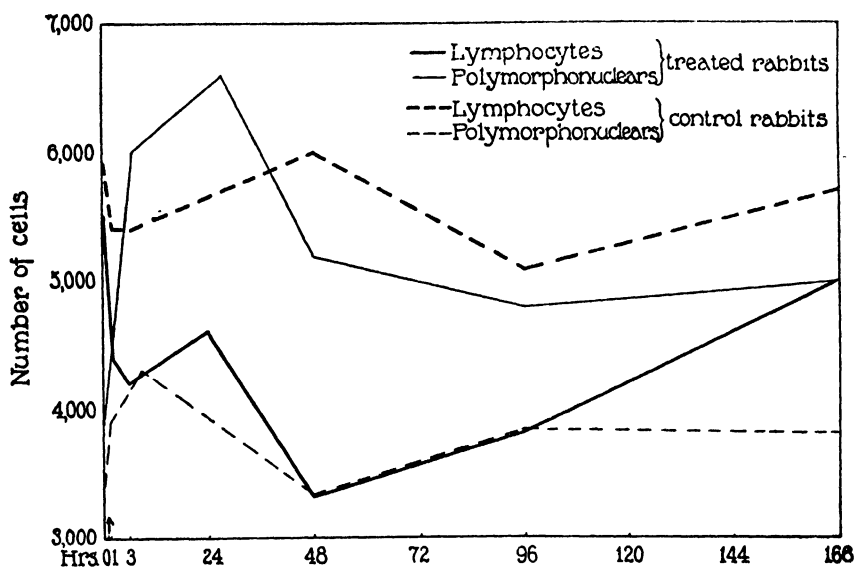
TEXT-FIG. 3.
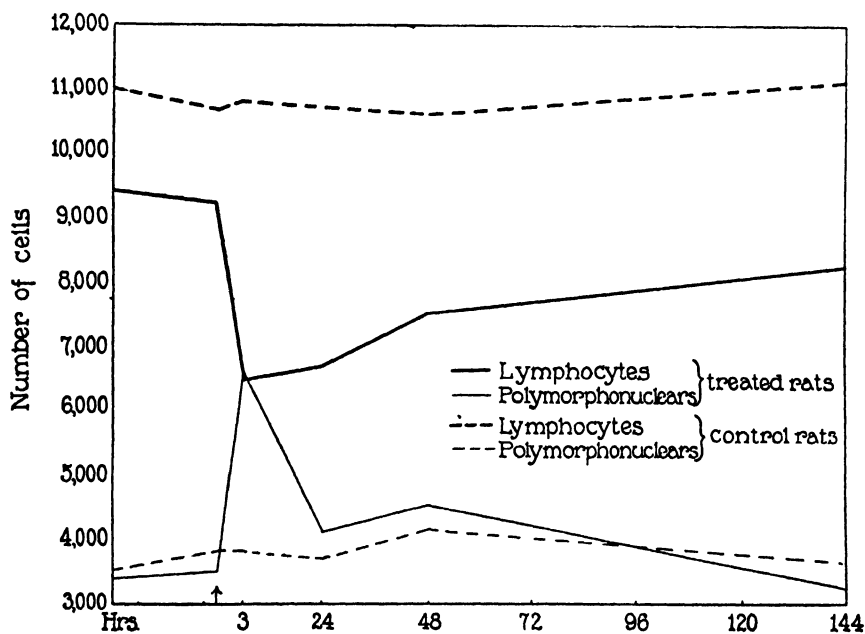
TEXT-FIG. 4.

TABLE IV.

Effect of Urethane on the Leucocytes of the Blood of Rats.

No.	0 hr. L	0 hr. P	0 hr. L	0 hr. P	3 hrs. L	3 hrs. P	24 hrs. L	24 hrs. P	48 hrs. L	48 hrs. P	144 hrs. L	144 hrs. P
1	9,400	3,000	9,900	3,100	6,300	8,000	7,000	3,700	9,000	3,400	9,600	2,800
2	12,800	3,700	11,900	2,700	6,900	7,300	7,100	3,600	9,200	3,700	9,600	3,000
3	9,900	3,100	11,600	2,800	5,500	7,900	6,700	3,400	8,100	3,000	9,000	2,600
4	12,300	3,300	11,800	3,300	6,600	9,500	7,200	3,100	8,200	2,800	9,000	2,900
5	12,400	1,600	12,800	1,600	6,300	8,700	7,600	3,500	9,200	3,200	8,700	2,800
6	13,000	2,600	13,200	2,900	5,600	9,900	7,300	4,000	9,100	3,400	9,900	2,700
7	11,000	2,700	11,800	2,700	6,400	9,200	9,800	4,800	8,600	5,500	11,100	3,200
8	11,800	1,800	12,600	2,000	7,500	8,200	7,300	3,400	9,000	2,700	9,400	2,400
9	12,500	3,100	12,200	2,800	6,800	8,600	7,300	3,200	9,500	2,900	10,700	1,800
10	12,800	2,500	12,500	2,600	7,700	8,100	7,000	3,200	8,800	2,400	9,700	2,100
11	5,200	1,900	5,100	1,700	6,100	4,300	8,100	5,400	7,800	4,000	6,200	2,700
12	8,100	3,100	7,400	2,900	7,100	4,900	5,500	3,900	6,400	3,000	4,800	2,800
13	7,400	3,500	7,800	2,500	3,200	7,100	6,400	3,800	6,000	4,500	7,100	3,500
14	9,100	3,100	9,000	2,300	9,400	3,100	7,300	4,000	7,500	4,800	10,000	2,400
15	7,400	4,000	7,800	3,100	5,200	4,500	5,400	3,400	5,200	7,400	7,000	1,900
16	8,300	2,900	7,400	2,700	5,400	3,900	5,600	4,900	5,200	2,900	7,000	2,000
17	11,900	2,800	11,400	5,000	9,000	7,400	9,900	5,900	10,200	6,000	12,900	4,000
18	8,600	3,500	7,800	3,700	5,600	5,600	7,400	4,400	7,000	5,800	8,600	2,600
19	9,700	3,400	8,600	3,200	6,400	6,600	5,800	2,400	6,600	3,300	9,400	2,800
20	10,000	4,200	10,600	6,200	5,800	8,400	7,200	3,800	7,900	4,800	10,000	5,300
21	7,400	2,700	6,600	2,900	5,000	4,500	5,800	4,600	9,200	4,800	7,400	3,800
22	6,900	2,900	7,300	2,600	5,800	5,400	4,400	4,000	6,400	5,200	5,800	3,800
23	8,600	4,400	9,400	3,400	4,000	6,000	7,400	3,600	8,200	5,200	8,000	3,600

24	10,400	4,400	9,800	4,400	6,600	5,400	5,600	4,600	9,800	4,400	9,000	3,000
25	12,000	5,400	11,000	5,000	6,200	6,000	5,200	5,100	8,800	5,200	9,200	5,400
26	11,500	4,000	11,600	3,200	6,400	5,000	6,300	4,600	6,000	7,500	7,200	5,400
Average....	9,400	3,400	9,200	3,500	6,500	6,600	6,700	4,100	7,500	4,500	8,200	3,200
Controls.												
1	13,200	2,100	13,000	2,300	13,200	1,900	14,000	2,300	13,800	2,200	13,900	2,200
2	13,300	2,000	12,600	2,100	13,300	1,800	14,000	2,300	14,400	2,300	14,000	2,400
3	13,700	2,200	13,800	2,200	12,700	2,000	13,300	2,000	13,500	2,200	13,400	2,200
4	13,400	2,200	14,100	2,400	14,100	2,200	14,000	2,300	14,500	2,100	14,100	2,300
5	11,700	3,700	12,800	3,200	10,700	5,500	12,500	4,600	10,100	4,500	12,700	4,200
6	10,700	3,400	8,900	3,800	9,500	4,000	8,000	4,600	9,100	4,900	12,000	5,100
7	11,300	4,000	10,800	5,300	11,100	6,300	12,300	4,000	9,900	6,000	10,200	7,100
8	12,500	6,200	11,800	6,100	11,800	6,400	11,800	5,000	13,500	5,800	14,000	5,700
9	10,300	5,000	11,200	6,500	14,400	2,700	10,800	4,800	11,400	6,400	13,500	6,100
10	6,700	3,200	8,200	2,000	8,500	2,400	6,900	2,700	7,300	4,300	6,300	2,300
11	6,800	3,500	7,700	3,600	7,100	4,000	7,000	3,000	7,200	2,700	8,400	2,700
12	10,600	3,700	10,800	2,700	10,500	3,100	10,000	2,800	9,700	4,600	8,400	4,300
13	8,500	3,300	8,200	4,200	8,200	3,600	7,300	5,300	8,400	3,300	7,500	3,700
Average....	10,700	3,500	10,700	3,800	10,800	3,800	10,700	3,700	10,600	4,100	11,100	3,600

The average of these counts showed a decrease in the lymphocytes of about 50 per cent in 3 hours after the urethane, which persisted for 48 hours with an approximate return to the normal in 6 days. The polymorphonuclear leucocytes increased rapidly after the injection of urethane reaching a maximum of over 200 per cent at 3 hours and then slowly returned to normal.[7]

The marked and persistent alkalosis of the blood associated with urethane anesthesia eliminates the use of this agent in all experiments designed to study the acid-base equilibrium of the blood.

DISCUSSION.

The blood cell changes accompanying urethane anesthesia are of interest on account of the possible association of alkalosis in general with decreases in the lymphocytes. Work from this laboratory[6] has shown that a dose of x-ray sufficient to destroy the lymphoid tissues brings about a state of uncompensated alkalosis while the same degree of alkalosis when induced by the injection of sodium bicarbonate is accompanied by an identical change in the blood picture. These three observations are suggestive of a possible association between the reaction of the blood and the cell content but the processes involved are so complicated that perhaps even a tentative deduction would be ·unwarranted However, the marked effect of urethane anesthesia revealed by the blood counts may explain some of the prevailing differences of opinion as to the action of x-ray on the circulating lymphocytes, since this method of anesthesia has been extensively used by some of the workers experimenting with x-ray exposures.

CONCLUSIONS.

When anesthesia is produced in rabbits or rats by injections of ethyl urethane, the CO_2 content and pH of the whole blood of the animal are more or less rapidly increased to a point where there is a marked uncompensated alkalosis which reaches its maximum at 24 hours and persists for 48 hours.

The increase in the CO_2 content and pH of the whole blood is accompanied by a decrease in the circulating lymphocytes and an increase in the polymorphonuclear leucocytes.

[7] It is of interest to note that, in some preliminary experiments, rats under urethane anesthesia, given an exposure to x-ray well below the lethal dose, invariably died between 7 and 14 days later.

PRIMARY LUNG TUMORS IN MICE FOLLOWING THE CUTANEOUS APPLICATION OF COAL TAR.*

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 35 AND 36.

It has been a not unusual observation in our experience that mice failing to develop skin cancer as the result of the application of coal tar are found to have tumors of the lungs. The first explanation of the pulmonary tumors which suggests itself is that a cancer of the skin had occurred as a result of the tar, had given off a metastasis to the lung, and had then either healed or sloughed out. But frequent and careful examination of the animals during life failed to show any lesions of the skin suggesting cancer, and at autopsy there were no scars or other evident abnormalities in the painted areas. Furthermore, histological examination of the nodules in the lungs revealed a structure and a type of cell different from the metastases which occur from the tar skin cancers. Spontaneous tumors in the mouse lung have been frequently observed but they rarely occur in such young animals or in such a high proportion of individuals as in our experiments. In the following experiments we have attempted to eliminate completely the probability of skin lesions while determining the influence of the external application of tar on the incidence of primary tumors of the lungs.

Experimental Method.

It is well known that the induction of skin cancer in mice by the application of coal tar requires frequently repeated applications of this agent. With the product used in this laboratory three applications weekly for 3 months suffice to induce cancer in a fair proportion

of the animals.   When a high rate of incidence is desired it is neces-
sary to continue the paintings for 4 months.

Tar Preparation.—The tar product used throughout these experiments had
for its base the residue from a coke oven in which the crude tar had been distilled
at a temperature of approximately 377°C.[1]   This hard residue was pulverized
and extracted with benzene.   The soluble fraction was concentrated, washed
first with 12 per cent NaOH, and then with 30 per cent H_2SO_4.   The precipitates
were removed and the benzene fraction was then evaporated down until all of the
excess benzene had been removed.[2]

Method of Application.—The tar was applied by means of a swab to an area
of skin somewhat less than a centimeter in diameter.   There were twelve such
areas on the surface of each mouse, painted in rotation according to the following
scheme.

Area.	Days of application of tar.			Area.	Days of application of tar.		
1	1	29	57	7	15	43	71
2	3	31	59	8	17	45	73
3	6	34	62	9	20	48	76
4	8	36	64	10	22	50	78
5	10	38	66	11	24	52	80
6	13	41	69	12	27	55	83

By this method each mouse received thirty-six applications of tar
distributed over 83 days.   Each individual area received three appli-
cations during this time with practically a month between each paint-
ing.   The selection of the areas and the order in which they were
painted were so arranged as to avoid the possibility of overlapping of
tarred spots.   The location of the areas and the order in which they
were painted are shown in Text-fig. 1.

[1] This material was kindly furnished by the Barrett Company.

[2] For details of preparation see Murphy, Jas. B., and Landsteiner, K., *J.
Exp. Med.*, 1925, xli, 807.

Experiment 1.—20 mice from 3 to 4 months old, selected from a fairly uniform stock with an extremely low cancer incidence, were painted with tar according to the above scheme.   Of these 12 survived for from 1 month to 6 months after the tar paintings had been stopped.   When killed 10 of the 12 showed one or more pearly white nodules in the lungs varying in size from 1 to 4 mm. in diameter. There was no detectable change in the skin of the painted regions.   Microscopic examination of the lung nodules showed them to be typical epithelial tumors in eight of the specimens.   In the other two instances the nodules were lost.   The

Areas painted with tar
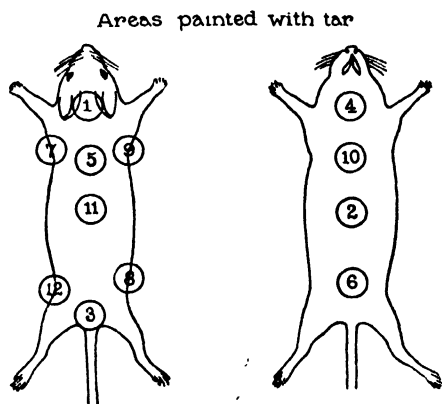
TEXT-FIG. 1.

lung tumor rate for this experiment was 66.6 per cent, as confirmed by histological examination, and 83.3 per cent if the 2 animals are included in which the diagnosis was made only on the gross specimen.

As a control to this experiment 22 mice from the same stock ranging in age from 8 months to a year, and kept under the same laboratory conditions, were killed. Careful examination of the lungs failed to show a single instance of tumor.

Experiment 2.—A second group of mice was subjected to the same system of tar applications as that used in Experiment 1.   All but 5 of the animals were lost in an epidemic early in the experiment.   The surviving animals were killed 6 months after the last tar application and 3 of the 5 were found to have tumors of the lungs, confirmed by microscopic examination, and 1 a tumor of the uterus. The tumor incidence for this experiment was thus 80 per cent and there were lung tumors in 60 per cent.

Experiment 3.—This group was made up of 40 mice between 2 and 3 months old, selected from a fairly uniform stock known to have a low tumor incidence. The animals were painted with tar in the same fashion as in Experiment 1. About 4 months after the last application 23 of them were killed. The skin showed no alteration in the painted regions. In 2 animals the lungs were normal but in all of the remaining 21 there were pearly white nodules in these organs. As many as 15 such tumors were found in the lungs of 1 animal. In diameter they varied from 1 to 4 mm. (Text-fig. 2). Microscopic examination showed the growths to be epithelial tumors of the same type as those in the preceding experiments in 18 of the 23 animals. The age of the mice at the time they were killed was between 9 and 10 months.

For controls a group of 16 untreated mice from the same stock, but from 1 to 6 months older than the experimental animals, was kept under the same conditions and killed at the same time. The age of these mice at the time they were killed ranged from 12 to 16 months. In not a single instance was a tumor of the lung or other organ encountered. The tumor incidence for the painted animals was in this experiment 78.3 per cent as compared with 0.0 per cent for the controls.

Character of the Tumors.—The cells making up the tumors are fairly large, cuboidal or ovoid in shape, usually lying in a single layer on either side of a thin shred of stroma (Figs. 1 to 4). The general pattern gives the impression that the tumor is composed of closely packed folds of an epithelial cord. There is some variation in the density of the tumors and in the amount of stroma, but the general histologic type is uniform. Mitotic figures are present in fair number. As a rule the tumors are sharply demarcated from the surrounding lung tissue but it is not uncommon to find finger-like processes invading the lung tissue. Occasionally there is some reaction at the edge of the growth and an inflammatory condition in the neighboring lung tissue but this is an exceptional finding.

The growths are identical in structure with those described as primary tumors of the lung in mice by Levingood,[3] Haaland,[4] Tyzzer,[5] and Murray.[6] They have been variously designated as adeno-

[3] Livingood, L. E., *Bull. Johns Hopkins Hosp.*, 1896, vii, 177.

[4] Haaland, M., *Ann. Inst. Pasteur*, 1905, xix, 165.

[5] Tyzzer, E. E., *J. Med. Research*, 1907–08, xvii, 155.

[6] Murray, J. A., *3rd Scient. Rep. Imperial Cancer Research Fund*, London, 1908.
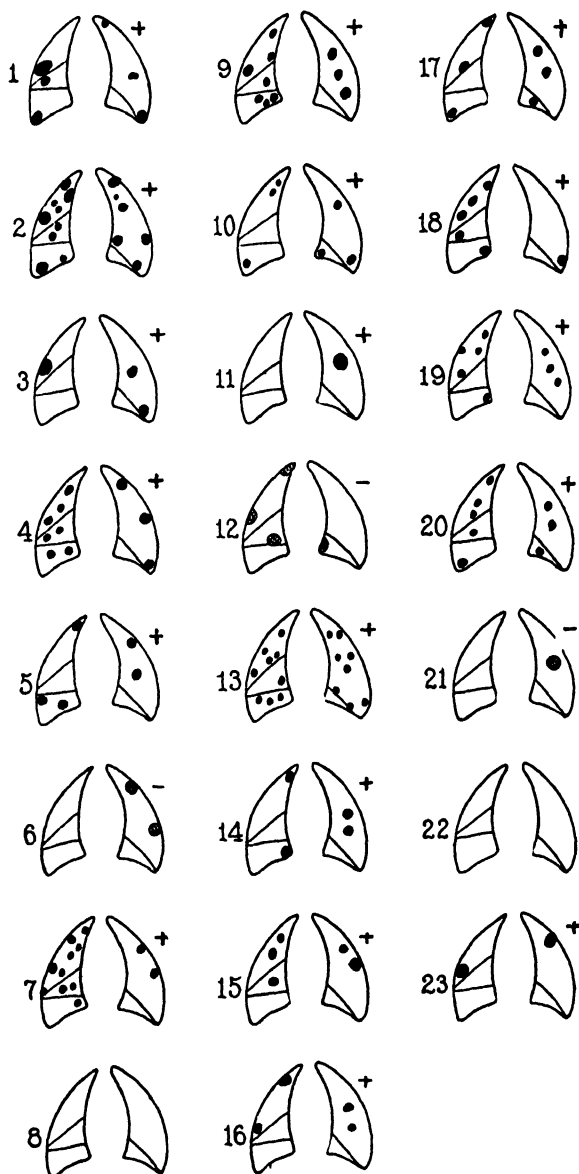
TEXT-FIG. 2. Schematic representation of the relative size and number of tumors in the lungs of mice from Experiment 3. The solid black areas indicate that the tumors had the gross appearance of new growths while those cross-hatched were of doubtful nature. The + indicates that a neoplasm was present on microscopic examination and − that no evidence of tumors was found.

carcinoma, papillary cystadenoma, and adenoma. However, judging by the descriptions and published illustrations there is but little variation amongst them and they unquestionably represent a distinct type of epithelial tumor.

DISCUSSION.

As already stated, spontaneous tumors of the lung are not uncommon in certain strains of mice. Tyzzer[5] has reported a high incidence in one of the groups he studied. Slye[7] has noted such tumors in some of her strains. In our tumor stock it is rare to find a lung tumor in animals 12 months old or younger, while the highest rate comes between 26 and 29 months of age.[8] In the case of two special strains, with a lung tumor rate between 59 and 70 per cent, in only one instance has a tumor occurred in a mouse as young as 13 months while the average age is about 20 months. With these facts before us we have attempted to eliminate as completely as possible the chance of spontaneous tumors from our experimental material. In Experiment 1 all but four of the mice were killed before they were 11 months old and the remaining four were killed at 13 months. In Experiment 3 all of the animals were from 9 to 10 months old at the time they were killed and the control mice were from 12 to 16 months old. Therefore it can be definitely said that all of the experimental animals were below the age at which spontaneous lung tumors occur in even a high tumor strain and that they were from a stock showing no lung tumors even at a more advanced age than the experimental animals.

The mechanism of the tar action is not clear. The experiment effectually eliminated the possibility that the lung tumors are in the nature of metastases from skin cancers. Two other possibilities may be considered; i.e., that tar particles get into the lungs through the lymphatics; or that the tar painting so alters the body state that tumors occur at points of incidental irritation at which under ordinary circumstances they would not develop. The first possibility seems a little far fetched. The tar particles might be absorbed through the skin or the alimentary canal since some of the tar is licked off by the animals. In either case the tar would have to pass through at least

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[7] Slye, M., Holmes, H. F., and Wells, H. G., *J. Med. Research*, 1914, xxx, 417.
[8] Observations in process of publication.

one set of lymph glands in case it was to reach the lungs. The possibility of the inhalation of particles can be eliminated as the tar is usually either licked or rubbed off before it dries. When some does adhere it becomes very hard and is powdered with difficulty.

In support of the second hypothesis we have accumulated a large amount of data which go to show that the repeated application of tar greatly reduces an animal's resistance to transplanted cancer.[9] Animals of tested resistance, either natural or induced, may be rendered susceptible to a subsequent transplant by tar application. Granting that the application of tar reduces the general resistance to cell growth, is there any evidence that the lungs are the site of irritant processes such as might conceivably act to precipitate the development of neoplasms? Our mice lived in burrows under sawdust and shavings mixed with chopped hay. There is every probability that the inhalation of some small foreign particles resulted in irritation. As a matter of fact inflamed areas are not uncommon findings in the lungs of mice. This hypothesis seems to us the most feasible but further work would be required to establish it.

SUMMARY.

The external application of tar to a number of separated areas on the surface of mice, in such fashion that no single area is irritated sufficiently long to cause lesions of the skin, has resulted in a very high incidence of lung tumors. This incidence ranged from 60.0 per cent in one experiment to 78.3 per cent in another. Control mice from the same stock but from 3 to 6 months older, and for that reason the more liable to spontaneous lung tumors, failed to show a single instance of such growths. Even in a stock in which spontaneous lung tumors had been frequent, the incidence for corresponding age periods has never been above 5.5 per cent while the average has been between 1 and 2 per cent over a period of years. The tumors in the tar-painted animals occur as small white nodules, either single or multiple. They are typical epithelial neoplasms, identical histologically with those described by previous authors as occurring spontaneously in mice.

Some possible factors in the causation of the tumors are briefly discussed.

[9] The data will be published in full in a subsequent article.

EXPLANATION OF PLATES.

PLATE 35.

FIG. 1. Low power photomicrograph of a section through the lower lobe of the lung of a mouse which had received external applications of tar.

FIG. 2. A photomicrograph of an earlier stage of a primary tumor of the lung following external applications of tar.

PLATE 36.

FIG. 3. A primary tumor of the lung following external applications of tar.

FIG. 4. A high power photomicrograph of a lung tumor in a tar-painted mouse, showing mitotic figures.
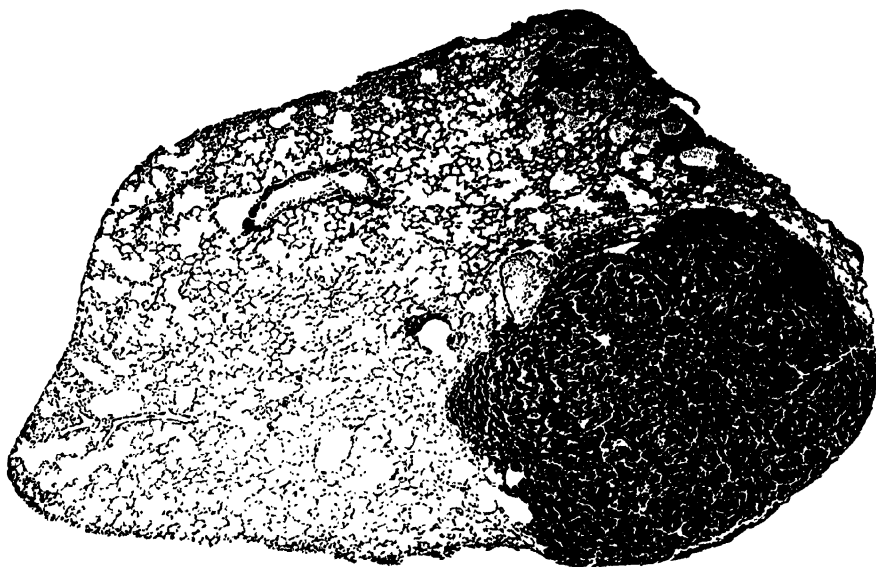
Fig. 1.
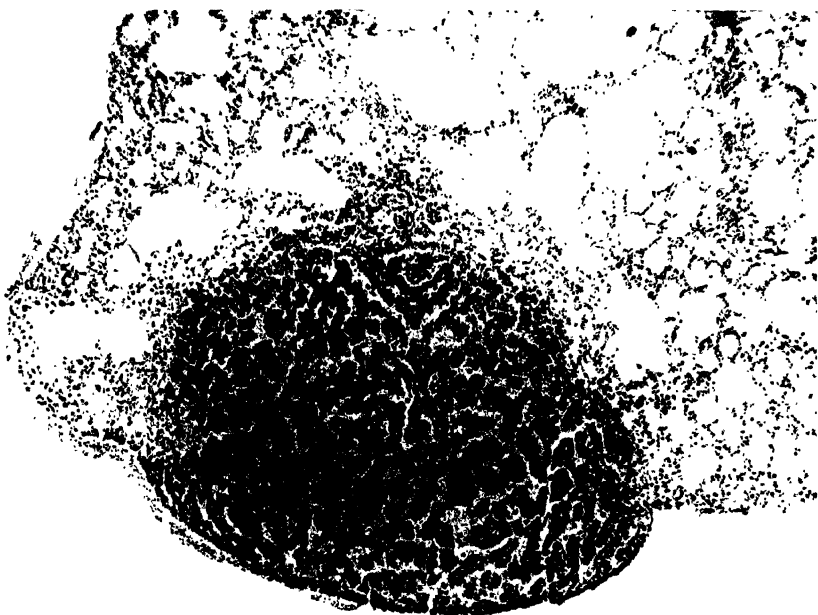
Fig. 2.

(Murphy and Sturm: Primary lung tumors in mice.)
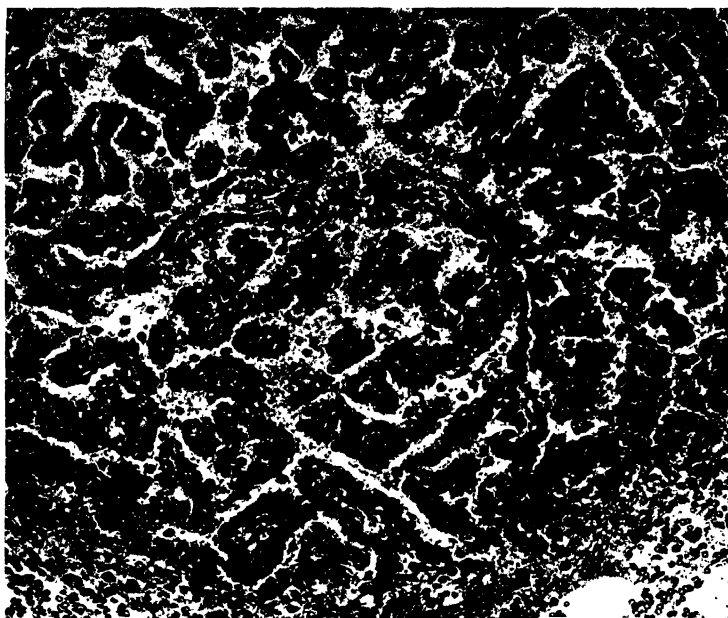
Fig. 3.
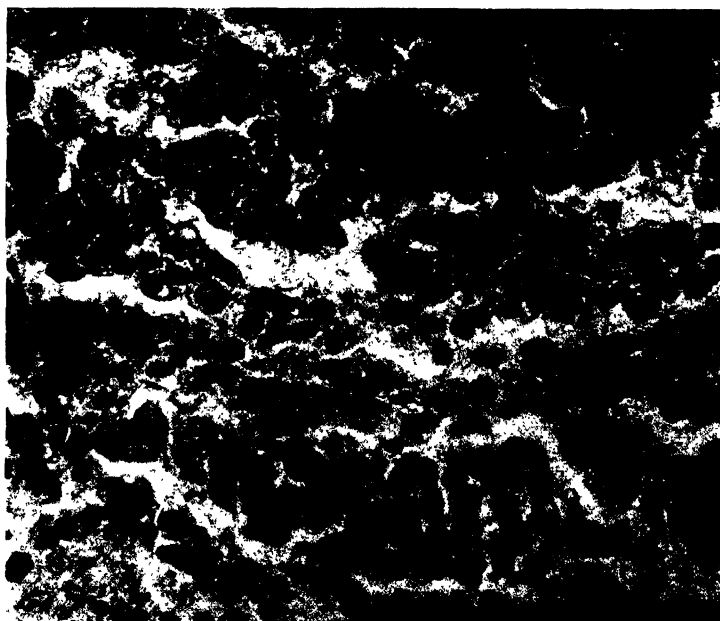
Fig. 4.

(Murphy and Sturm: Primary lung tumors in mice.)

SKIN REACTIONS TO ALTERNATE HEAT AND X-RAY EXPOSURES.*

By JAMES A. HAWKINS, PH.D., AND HARRY CLARK, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

We have previously reported some observations on the effect of simultaneous exposures to heat and x-rays on the skin reactions of guinea pigs.[1] The dosage of x-ray used in these experiments when given alone was not sufficient to cause even a mild erythema, while the heat exposure alone gave only a slight burn in 50 per cent of the animals. However, when the two agents were applied simultaneously, well marked burns resulted which healed very slowly. The time at which the burn developed and the character of the first stages were similar to a simple heat effect but the later appearance of the lesions and the slowness with which they healed suggested an x-ray burn.

It was the purpose of the present investigation to determine whether or not one of the physical agents mentioned is capable of sensitizing the tissues to the action of the other.

Method.

The character of the x-ray and the method of application have been previously described.[1,2] During exposure the animal and x-ray tube occupied separate compartments of a lead-lined cabinet with an aperture for the passage of the rays, closed with thin bristol board. Both chambers and the partitions were kept cool and ventilated by means of an electric fan. These precautions prevented any heat from the tube penetrating to the animal. The dosage used throughout the experiments was supplied by an outfit known to produce x-rays remarkably constant both in quality and intensity. The tube was operated at 30 kilovolts

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[1] Hawkins, J. A., and Clark, H., *J. Exp. Med.*, 1925, xli, 761.
[2] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

and 22 milliamperes with a target distance of 27.5 cm. The duration of exposure was 11 minutes, giving a dosage which previous experiments had proved to be suberythemal.

The technique for holding the animals in place and at a definite target distance has already been described.[2]

Heat treatments were given by means of a flat hollow brass button, 3/4 inch in diameter and 3/8 inch thick, through which water from a constant temperature bath was passed. The circulation from the bath through the button and back again to the bath was maintained by means of an electric pump driven at a definite speed. The temperature of the water fell less than 0.5°C. in passing through the circuit. When in use the button was attached firmly to the abdomen of the animal by means of a broad elastic band which encircled the body. The heat treatment was thus confined to the portion of skin covered by the button. Throughout the experiments the temperature of the water in the button was maintained at 46°C.

Experiment 1.—The abdomens of twenty guinea pigs were shaved and an area exposed for 11 minutes to x-rays of the intensity noted above. The brass button was then placed over part of the same area and water at 46°C. was circulated through it for 10 minutes. The heat in the same dosage was next applied to another area on the same animal but this area had received no previous exposure to x-rays. Thus the control exposures to x-rays and heat alone and the combined exposures to the two agents were made on each animal.

A slight scaling of the skin resulted from the heat exposure, which appeared about the 2nd day and lasted from 4 to 8 days (Text-fig. 1). In the areas exposed first to x-ray and then to heat scaling developed on the 2nd day and definite burns appeared on the 4th. The healing of these burns was somewhat irregular. In sixteen pigs it was completed between the 15th and 19th days, but in the remaining four the lesions were not covered till between the 32nd and 39th days (Text-fig. 2). The areas exposed to x-ray alone developed slight scaling of the skin on about the 7th day after treatment and this persisted for from 4 to 5 days (Text-fig. 3).

Experiment 2.—Heat was applied for 10 minutes to two widely separated areas on the abdomen of each of nineteen guinea pigs by means of the brass disc with the water circulated at a temperature of 46°C. Then an area on the abdomen was exposed to x-radiation so that one of the previously heated spots was included in the x-rayed area. The dosage was the same as that used in Experiment 1. Thus we had on each animal an area exposed to heat alone, x-ray alone, and o exposed first to heat and then to x-ray.

The results obtained in this experiment were identical with those in Experiment 1 and are shown in Text-figs. 1 to 3.

From these experiments it is seen that there is a very marked difference in the reaction of the areas which received either x-ray or heat alone and those exposed to both types of radiation in sequence. The maximum effect of either agent alone was a slight scaling of the
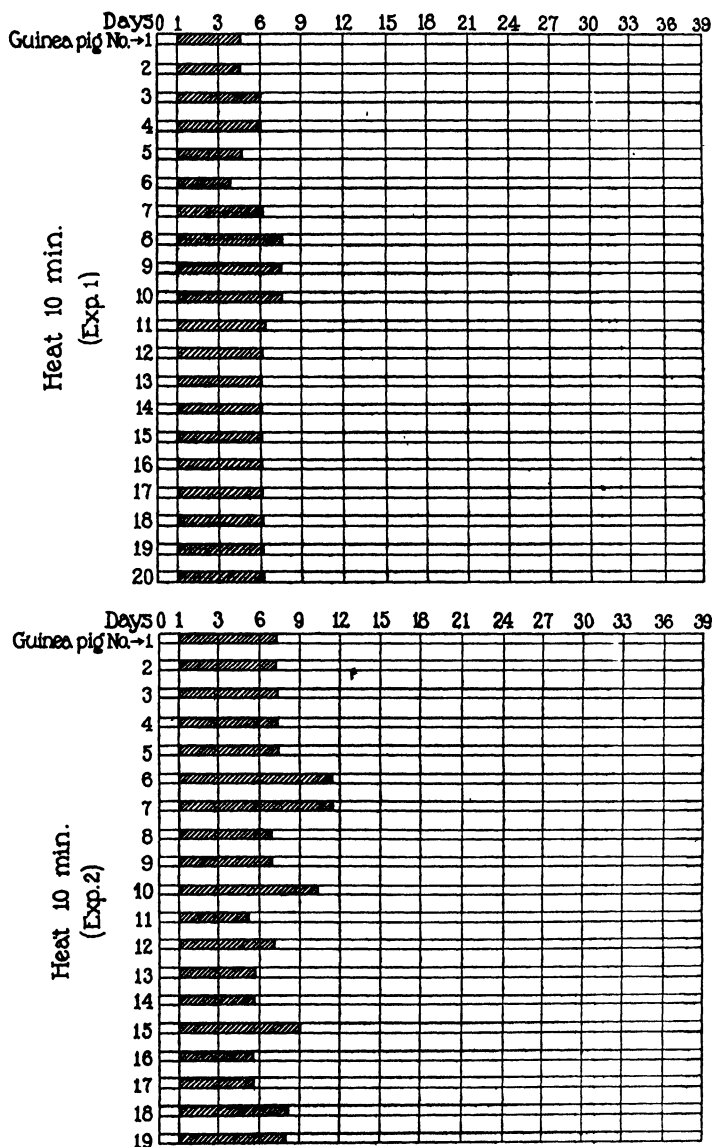
TEXT-FIG. 1.

TEXT-FIGS. 1 TO 3. Each line represents the history of a single individual area from the time of exposure until the end of the experiment. The heavy lines indicate burns; the cross-hatch indicates scaling but otherwise intact skin; and the unshaded area indicates that there was no reaction or else a healed lesion in the skin.
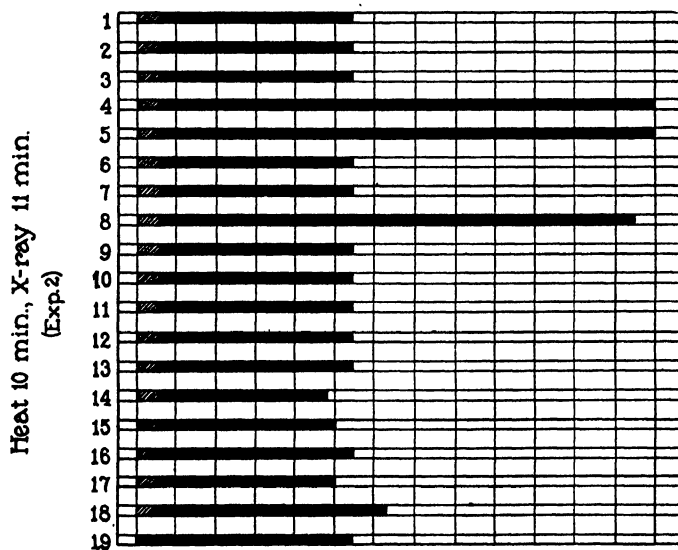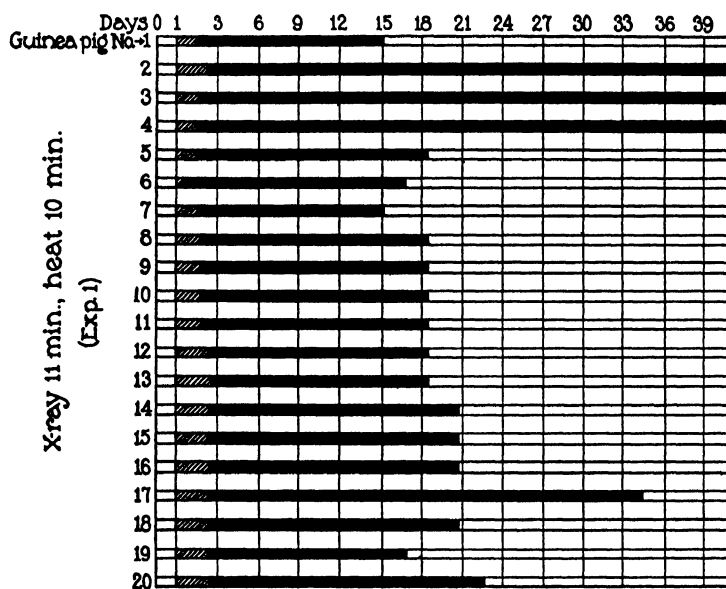
TEXT-FIG. 2.
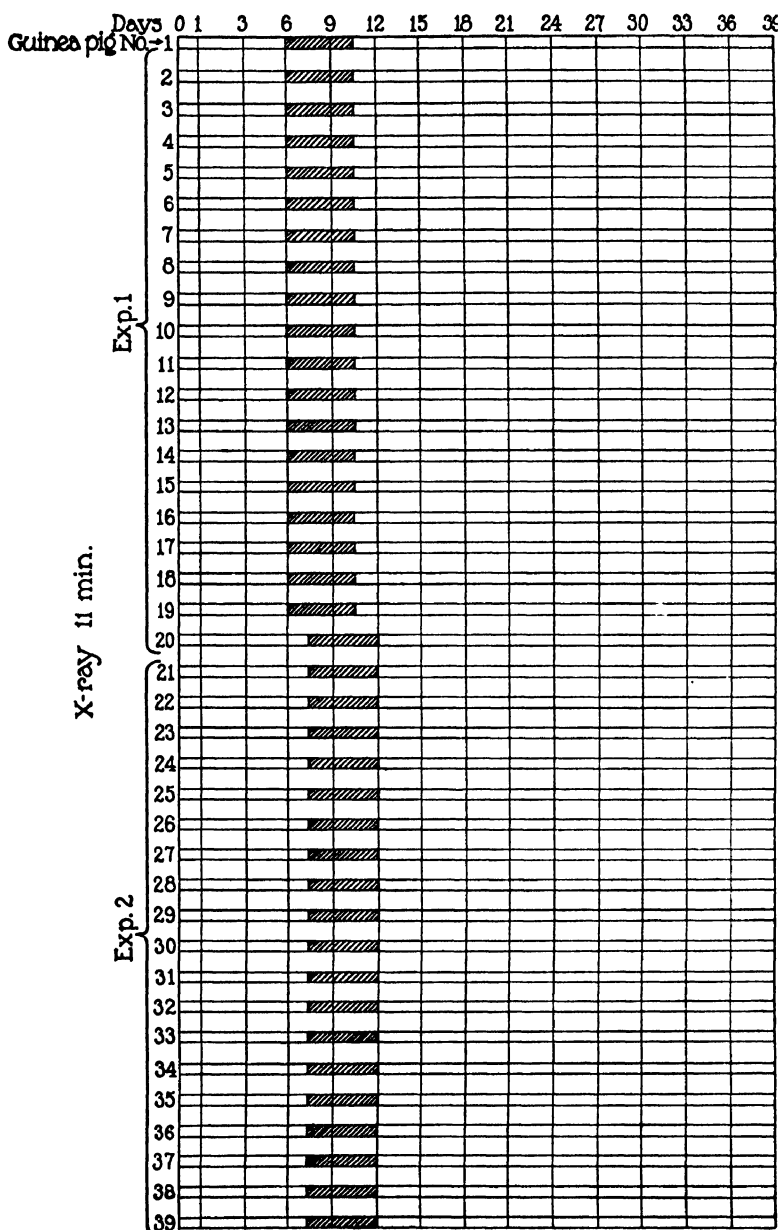
TEXT-FIG. 3.

skin.   In the areas exposed to both agents a burn invariably resulted. It seemed to make no difference in the intensity of the reaction whether the heat was followed by the x-ray exposure or the x-ray followed by the heat exposure.   The burns appeared as white spots corresponding in size to the area covered by the brass disc.   They became evident on the 2nd day after treatment and broke down usually on the 4th day.   The majority of the lesions healed in from 15 to 18 days, leaving a thick scar.   In about 50 per cent of the animals, some weeks later, the areas broke down a second time with the formation of chronic ulcers which healed very slowly.   It is apparent from these observations that either type of radiation is capable of augmenting the action of the other.

DISCUSSION.

The mechanism in the cell which brings about the response to radiant energy is not understood.   Bovie,[3] in the discussion of his observation that ultra-violet light sensitized paramecium to heat, suggests that the radiation induces some rearrangement of the atoms within the cell so that they may change from a passive to an active stage. The process of rearrangement by which the complex cell protoplasm returns to the normal equilibrium after x-ray exposure is undoubtedly slow.   The heat radiation may intensify the atomic chaos so that the cell is destroyed before adjustment can take place.   In the case of the present results the reaction was the same regardless of whether the heat or x-ray exposure was made first, in which respect they differ from Bovie's observation that ultra-violet light sensitizes to heat applied later, whereas the application of the two agents in reverse order is without effect.

The nature of the lesions produced in our experiments suggests the effect of both agents.   The fact that they develop so soon after treatment and their general appearance during the first few days give them a close resemblance to a pure heat effect, but the later appearance of the burns, the slowness of healing, and the type of resulting scar are more characteristic of an x-ray effect.   The observations would seem to be capable of further analysis with a possibility of yielding some insight into the nature of the effect of radiant energy on the living cell.

[3] Bovie, W. T., and Daland, G. A., *Am. J. Physiol.*, 1923, lxvi, 55.

SUMMARY.

Different areas on the abdomen of the same guinea pigs have been exposed to suberythema doses of soft x-rays, to heat of an intensity below the critical dose for the production of burns, and to both radiations in sequence.

The only effect of exposure to x-ray or heat alone was a slight scaling of the skin. The areas exposed to heat and x-radiation developed well marked and persistent burns. The results were the same no matter in which sequence the agents were applied.

STUDIES ON THE RELATION BETWEEN TUMOR SUS-
CEPTIBILITY AND HEREDITY.

II. The Incidence of Tar Tumors in Strains of Mice Having a
Differing Incidence of Spontaneous Growths.

By CLARA J. LYNCH, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The ability to produce malignant new growths at will in laboratory
animals has provided a new and valuable instrument for the investiga-
tion of cancer. Fibiger's[1] success in causing neoplasms in the stomach
of rats infected with Spiroptera was followed shortly by Yamagiwa
and Ichikawa's work[2] with coal tar upon rabbits; and the application
of the coal tar method (Tsutsui[3]) to mice, the species in which trans-
planted and spontaneous tumors are best known, has opened the way
to one of the most popular fields of present day medical research.
The progress of the growths, the histological findings, the conditions
of occurrence and so forth, have been treated in many papers. In all
the published accounts it may be noted that there were wide variations
in the response given by the animals subjected to treatment.

It is difficult to compare results from different laboratories. Never-
theless the reports indicate that the same tissue in different species
shows different degrees of reaction to the treatment designed to elicit
neoplasms. The skin of rabbits and mice responds rather readily to
irritation with tar, while that of rats and guinea pigs appears to be
highly resistant. Not only are species differences indicated but among
individuals of the same species a wide range of variability is seen.
Apparently no tests have been made heretofore to determine whether

[1] Fibiger, J., Berl. klin. Woch., 1913, 1, 289; Compt. rend. Soc. biol., 1920, lxxxiii,
1160.

[2] Yamagiwa, K., and Ichikawa, K., Mitt. med. Fakult. k. Univ. Tokyo, 1915–16,
xv, 295; 1917–18, xix, 483; J. Cancer Research, 1918, iii, 1.

[3] Tsutsui, H., Gann, Japan Z. Krebsforsch., 1918–19, xii, 17.

the individuals manifesting distinct differences may not come from families which have peculiar and characteristic grades of susceptibility.

For some years there have been reported strains of mice which show differences, apparently hereditary, in the rate of incidence of spontaneous tumors, notably those of the mammary gland. Two such strains bred at The Rockefeller Institute seem to afford suitable material for tests as to whether there are hereditary differences affecting the response to treatment with coal tar.

EXPERIMENTAL.

The stocks chosen were a strain showing a high tumor incidence, No. 1194 from the Lathrop mice, and a strain exhibiting a lesser incidence, which was originally obtained from Dr. Bagg at the Memorial Hospital. Strain 1194 comprises strong healthy mice, rather large and of the wild, or agouti, color. Of the bred females reaching the age of 6 months or more, 65 per cent develop mammary gland tumors while those not bred develop them in 20 per cent. The Bagg mice are albino but are also a vigorous stock, in which the animals producing young have a 28 per cent tumor rate and those not bred only 4 per cent. The average tumor age of Strain 1194 is 12 months and of the Bagg mice 18 months.

The technique used was to paint the mice three times weekly for 4 months on a spot between the shoulders, applying approximately 1 drop from an applicator dipped in a solution made from coal tar residue. Depilation was found unnecessary but the hair was clipped before the first painting.

The tar solution used was a benzene extract free from acids, bases, and phenols made from the residue from a coke oven in which the tar had been distilled at a temperature of approximately 377°C. The residue was obtained from the Barrett Company who have very kindly cooperated with us a number of times by providing both residue and extract. We wish to extend our thanks to them.[4]

For the experiment 20 mice from each strain were selected. Approximately half were males and half were females that had not been bred. Tar was applied for 4 months, from the middle of November to the middle of March. The first painting was usually followed by a loss of hair from the treated area. Desquamation could be observed sometimes after a few paintings but the first papillomatous elevation was not noted until after the 90th day. The elevations were strongly keratinizing, often producing horns of remarkable size similar to those pictured by other investigators. When carcinomatous changes occured they became evident about the 140th day or later, either as an induration at the base of the horn or, if the latter had not persisted, at its former site. Occasionally they appeared without the formation of a papilloma. Experience has shown that the induration

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[4] For a more detailed account of the preparation of the tar product see Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 807.

Text-Fig. 1.

referred to is indicative of carcinomatous transformation of the cells but the diagnosis was confirmed by sections taken at the time of death.

In general the results of the experiment show that no significant difference in the response to tar painting was given by the two strains of mice with differing rates of incidence of spontaneous tumors.   Of the 20 mice from the strain with high incidence, all of which survived the period of painting (120 days) 12 eventually developed carcinomas of the skin (60 per cent); from the low tumor strain, the albinos, 19 individuals lived 120 days and again 12 produced carcinomas, approximately the same percentage.   Since the mice lived differing lengths of time, it is important to analyze the results more closely.   The course of the experiments may be followed in the graphs.   The percentages
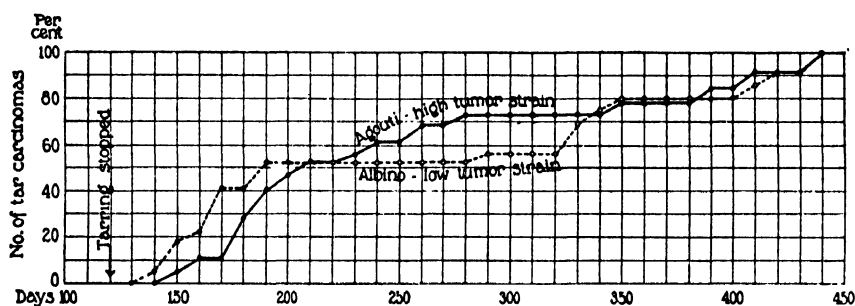
TEXT-FIG. 2.

from which the graphs were made, are based upon small numbers of individuals and therefore cannot be significant in detail but they are of value in indicating the general progress of events.

The histories of the individuals presented in the first two sections of Text-fig. 1 are condensed and compared in Text-fig. 2.   It will be seen that the carcinomatous transformations occurred slightly earlier in the albinos or low tumor strain than in the agoutis.   By the 140th day 1 of the 18 mice living at that period showed distinct induration while none of the high tumor strain did.   For a time the albinos continued to yield a larger number of individuals with carcinoma, showing at the 170th day 7 out of 17, or 41 per cent, as against 2 out of 18 or 11 per cent among the agoutis; but by the 210th day the percentages were even (9 out of 17 or 53 per cent in each case).   From the 190th

day the percentages are figured from the total number of carcinomas which had appeared in the group and the total number of animals living, or already dead, as a result of the neoplasm. From the 230th to 330th day the tumor rates of the agoutis exceeded that of the albinos but from the latter time to the end of the observations the rates were practically parallel. Three mice of the high tumor strain and five of the low exhibited a high degree of resistance for a considerable period but by the 440th day the sole survivor in each group had a cancer, but of very recent origin in one case.

We see then, that there was a gradual increase in the incidence of tar cancer in both strains, beginning with about the 150th day. The slight variations shown are not regarded as significant. It is to be noted in relation to the period of tarring that papillomas sometimes occur during the 4th month although they may not appear until after the applications have ceased. In the experiment now under consideration the carcinomatous changes took place only after the tarring was discontinued.

The series described were tarred during the winter months. In April an additional group of 10 albinos from the low tumor strain was subjected to the treatment. A few of these animals developed papillomas earlier (80th day) than either the agoutis or the first albinos and induration also was detected sooner in certain cases—in 2 of them before the end of the tarring (Text-fig. 1). Derom[5] has conducted experiments from which he concluded that the temperature of the tar and heating the tissues play a rôle in susceptibility to tar painting. The observations here reported are too meagre to be the basis of definite conclusions in regard to seasonal variations but they are not contradictory to Derom's point of view. Certainly they show the variability which may occur within a given strain and the inadvisability of using so called standardized controls.

The second albino group just mentioned was studied as an additional control for another series of mice. Those previously dealt with had been individuals from strains which produced tumors but which had not yet grown them themselves. It seemed pertinent to enquire

[5] Derom, E., Bull. Assn. franç. étude cancer, 1924, xiii, 422.

TEXT-FIG. 3

200
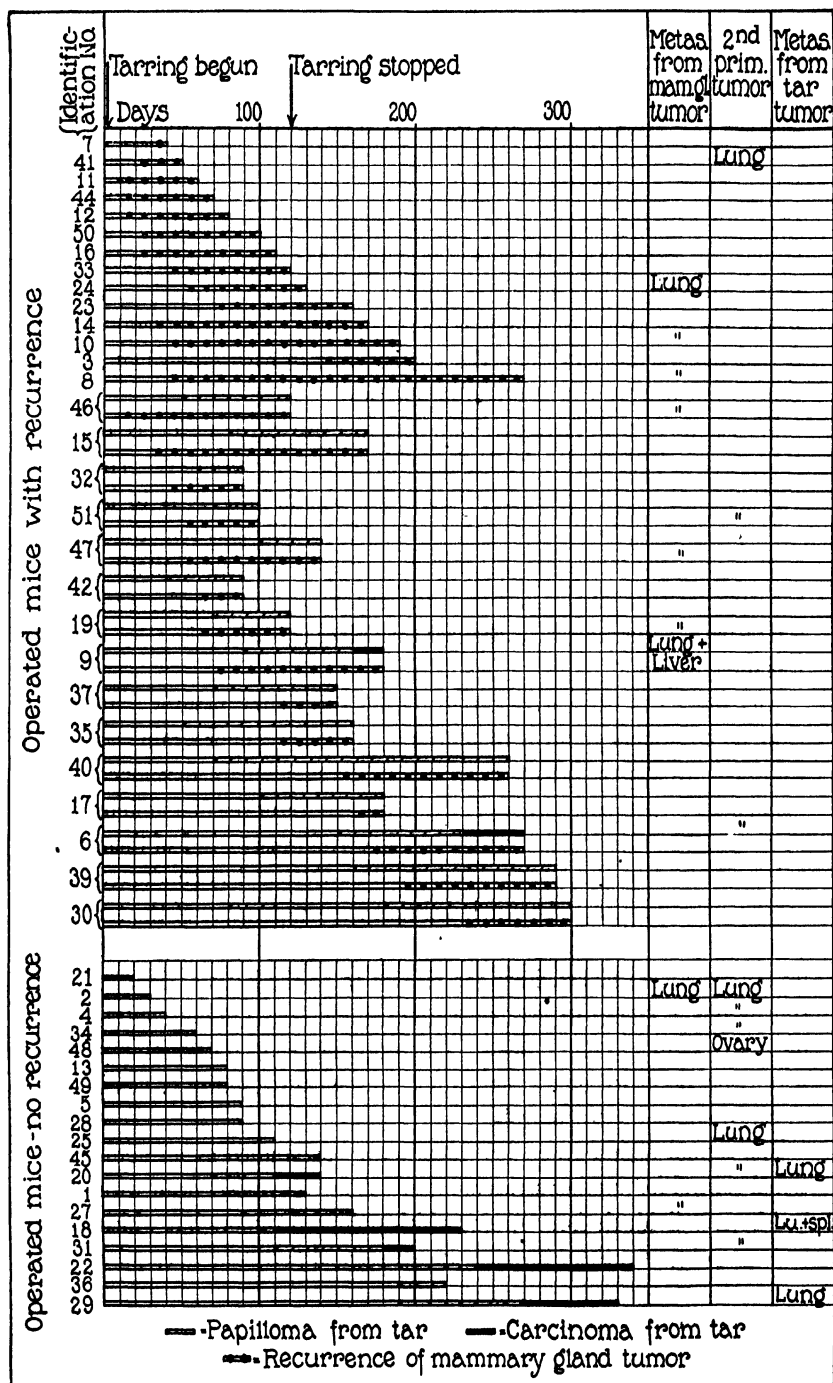
whether individuals which already had developed tumors would behave differently in response to tar.

Since mice with spontaneous tumors of the mammary gland rarely live more than 2 or 3 months, most of them could not be expected to survive the period of tar painting (4 months) unless the tumors were removed. It is difficult to obtain a large number of operable tumors at any one time, and so the collection of mice with suitable tumors was begun in the fall and continued several months. 48 of them were at length acquired, from a number of different strains. More than half were agouti; and most of the remainder were albino, though a few were chocolate, cinnamon, silver, or spotted. The tumor ablations were performed under ether by Dr. James B. Murphy and Mr. Ernest Sturm, to whom acknowledgement is here made. The growths were taken out soon after the tumor had been discovered. When the wound was healed and the mice in good condition, painting 3 times a week with coal tar extract was begun and continued for 120
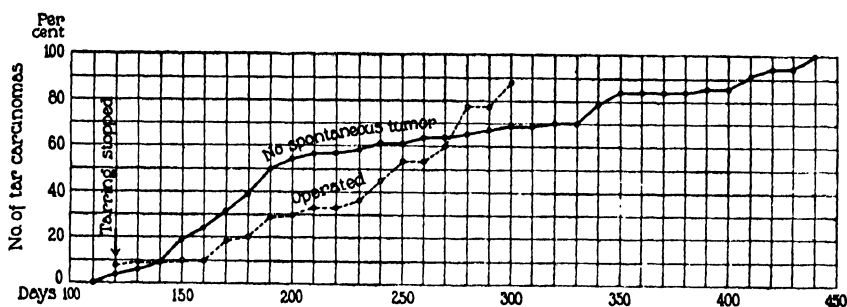
TEXT-FIG. 4.

days as in the experiment already described. With the mice first operated upon, treatment was begun in December, and the others were subjected to it at intervals, the last of them in April. This unavoidable spreading of the experiment over several months necessitated a second group of albinos as controls, tarred synchronously with the mice last operated upon, in addition to the groups with high and low tumor incidence, on which treatment had been started in November.

More of the mice submitted to operation died before the end of the tarring than of those not so treated even when the spontaneous tumor did not recur. By the 120th day only 28 of the mice operated upon were living compared with 49 in the group without spontaneous tumors. This was probably a result of the treatment received. The tumor mice were somewhat older than those without spontaneous tumors but not sufficiently so for age to have been an important factor

in the fatalities. Several of the mice operated upon developed papillomas after tarring earlier than did any in the control groups (50th to 70th day as against the 80th or 90th) (Text-figs. 1 and 3), and papillomas continued to be more frequent up to the 150th day. On the other hand the percentage of carcinoma developing in the operated series lagged behind that of the mice without spontaneous tumors until the 280th day. These differences are apparent from a comparison of Text-figs. 1 and 3. Text-fig. 4 affords a comparison of the percentage of incidence of carcinomas found at successive periods in the two groups of mice. The percentages are calculated as in the preceding graph. Although the series operated upon lagged behind the control the difference is not very marked. It will be seen that in general they behaved in a very similar way. If, however, we
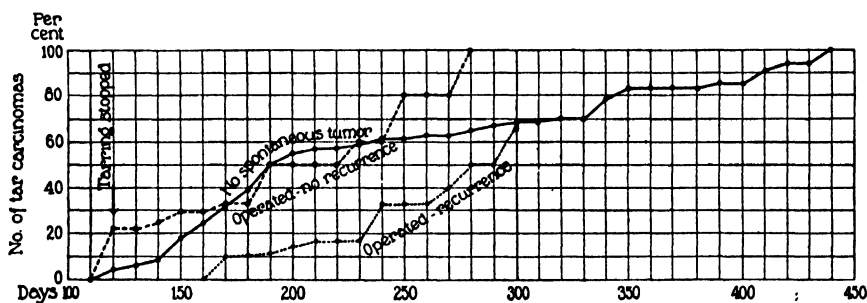
TEXT-FIG. 5.

distinguish in the series operated upon between those mice which did and those which did not have recurrences of the spontaneous mammary gland tumor, and compare the developments with those in the control mice, we find (Text-figs. 1 and 3) that it is the animals with recurrences which develop papillomas earlier, whereas malignant transformation rarely occurred in them. Of the 16 mice with recurrences which lived more than 120 days only 2 ever yielded carcinomas while 5 of the 9 which showed no recurrence eventually developed such growths. Text-fig. 5 shows the percentage of carcinoma at successive periods occurring among the three groups of mice: (1) those which had no recurrence, (2) those from which a mammary gland tumor had been removed and which did have a recurrence, and (3) those which had not developed a mammary gland tumor. The total number of

individuals in each group is very small so that the results are not conclusive but a difference in the response given by the mice with recurrences seems to be indicated. The first carcinomas in the series operated upon which showed no recurrences appeared at the 120th day when 2 of the 9 survivors (22 per cent) showed induration. In the non-operated group 2 carcinomas had appeared at this time (4 per cent) among the 49 surviving animals. None had occurred in the 19 mice operated upon which developed a recurrence then or later. Indeed, in the series last mentioned no carcinoma was found until the 170th day when 1 occurred in the group of 11 individuals as contrasted with 14 out of 44 (32 per cent) and 2 out of 6 (33 per cent) in the other 2 series. Unfortunately the mortality from the tarring was rather high among the mice from which the spontaneous tumors had been removed, so that even fewer individuals are represented in the later figures. However, the persistent absence of tar carcinomas in the mice with recurrences would seem important. At 230 days only 1 had died of such a carcinoma and 5 were still free from it (though 4 had papillomas), giving a group incidence of 17 per cent, whereas among those without recurrence 3 out of 5,—a total which includes both the living animals and those already dead from the neoplasm,—or 60 per cent, had developed a malignant growth. The control mice which had not been operated upon also showed a high incidence of malignancy, the record showing that 24 out of 41 of them (58 per cent) had tar tumors. At the 260th day there continued to be fewer tumors among the mice that had recurrences (2 out of 6) than among the mice without recurrences (4 out of 5) and also among the control animals (26 out of 41). By the 310th day all the mice with recurrences had died. The 2 mice without recurrences which were still living both had developed tar tumors.

The 2 oldest survivors among the animals with recurrences died after 290 and 300 days respectively without having developed more than a papilloma. Among the control animals 4 lived from 400 to 430 days from the beginning of the tarring without developing a neoplasm; but about the 410th day 1 of them developed a carcinoma where the tar had been applied, thus showing that a delay of as much as 290 days may elapse before there is a neoplastic response.

As already stated microscopic examinations were made of the

growths found at autopsy save in a few cases in which postmortem changes or other causes made it impossible. The malignant tar tumors proved to be carcinomas except in one instance (No. 18 in the operated group) which was a carcinosarcoma. Metastasis was found in 7 cases, usually to the lung but in No. 3, an albino, the mediastinum, diaphragm, and thoracic wall were also involved; and in No. 18 a metastasis from the sarcomatous portion of the tar tumor was found in the spleen. A number of tumors developed in regions outside the painted area. The only 2 of them in the mammary gland appeared in the agouti strain which ordinarily has a high incidence of mammary gland tumors. One was found in Mouse 7 which had not had a tar epithelioma, whereas the other developed in Mouse 2 about 100 days after the tar tumor had been first recorded. It is evident that a tumor produced by tar does not suffice to protect the host absolutely against the development of a spontaneous tumor in another organ. Male 13 which had developed in response to the tarring a small papilloma that persisted for only a short time, was very much later seen to have an infiltrating epithelioma of the jaw. Since mice remove tar from a painted area by licking it off it is perhaps surprising that epithelioma on the jaw or tongue is not more frequently encountered. In the case mentioned the neoplasm could not certainly be regarded as a result of the tarring since jaw tumors occur spontaneously in the strain. In two other cases the ovary was the site of a carcinoma but the most frequent region of occurrence for tumors developing at a distance from the tarred region was the lung. It is interesting to note that of the 23 pulmonary growths found none was in the agouti or high tumor strain in which the incidence of spontaneous lung tumors is very low.

SUMMARY AND DISCUSSION.

1. Two strains of mice, one with a high, the other with a comparatively low incidence of spontaneous tumors of the mammary gland, when painted between the shoulders with coal tar extract developed tar tumors with practically identical frequency.

Possibly this result was to have been expected. If tumors of certain organs or tissues are specific in heredity, a conception for which there is some evidence, then assuredly a high degree of incidence for one tissue, as for example the mammary gland, does not necessarily mean

a high incidence for another tissue, such as the skin, when subjected to tarring. To test the influence of heredity on the response of the skin to tar painting it would be best to employ strains of animals exhibiting differences in the incidence of spontaneous cancer of the skin. Unfortunately no such material was available. We can state on the basis of our experiments only that no hereditary differences in the response of the skin to tar painting were demonstrable in two strains of mice manifesting markedly different percentage incidences for spontaneous mammary growths. It is possible, of course, that the natural differences in the two strains may have been wiped out by the tar treatment. Such a view is suggested by the result of other work in this laboratory which has shown that tar painting increases markedly the incidence of tumors of the lung[6] and destroys the resistance to transplantable tumors (unpublished work).

2. Mice from which spontaneous mammary tumors had been removed were treated with tar. The percentage incidence of the resulting tar tumors was similar to that met with in the controls, except possibly in the case of such animals as showed a recurrence of the spontaneous growth. In them the development of tar tumors seemed to be delayed and possibly prevented. The numbers involved are too small to be conclusive.

Murray[7] has stated that "the induction of a fresh primary growth after a first has been definitely established, meets with a very intense resistance whether the first tumor be of the same parent tissue or another." His conclusions are based partly upon certain retarring experiments but more especially upon a series of mice from which spontaneous mammary gland tumors had been removed, which were then tarred. Apparently these latter did not have recurrences of the spontaneous growth. Only 1 of them developed a papilloma and none carcinoma. The tar was applied twice weekly in Murray's experiment instead of 3 times as in ours and for this as for many other reasons a direct comparison is not possible. It is furthermore not clear what the expectation for his group would have been. Our

[6] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1925, xlii, 693.

[7] Murray, J. A., *8th Scient. Rep. Imperial Cancer Research Fund*, London, 1923, 75.

conclusions, however, seem only partly to confirm his for we find that if any protection exists against a tar cancer after the ablation of a spontaneous mammary growth, it is only when a recurrence has taken place. From certain additional tarring experiments Murray concluded[8] that if a second tarring is begun before cancer has developed from the first, susceptibility is increased. Truffi[9] also has found a diminished refractoriness after tarring. Among our mice in which the mammary gland recurrence appeared during or after the tar painting, no increase in susceptibility but rather the reverse was found. Future experimentation must determine to which among several variables this divergence of results is to be attributed.

[8] Murray, J. A., *Brit. Med. J.*, 1924, ii, 1004.
[9] Truffi, M., *Riforma med.*, 1924, xl, 985.

THE CONFIGURATIONAL RELATIONSHIPS OF THE SUGARS, HYDROXY ACIDS, AMINO ACIDS AND HALOGEN ACIDS.

By P. A. LEVENE.

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In recent years, chemists have devoted much attention to the configurational relationships of monosaccharides, α-amino, α-hydroxy and α-halogen acids.

The field of chemistry in which the achievements of stereochemistry have been the greatest is undoubtedly that which deals with the configurational relationships of monosaccharides. The name of Fischer is preeminent among all workers in this field, but the name of Kiliani should also be remembered in this connection as he was the one to furnish a method for the synthesis of a higher monosaccharide from a lower one and Fischer made much use of this method.

The relationships of simple carbohydrates is a matter of common knowledge and need not be reviewed here. The most rational and clearest presentation of these relationships is found in the article of Rosanoff. A comprehensive summary on simple sugars in general is to be found in the monograph of Armstrong.

Fischer, in his day the greatest master of stereochemistry and also the greatest authority on sugar chemistry, was the first to point out the possibility of correlating the configuration of hydroxy acids with the configuration of monosaccharides. The pioneer work in this direction was done by Fischer himself and further progress was made by workers who received their training and inspiration from him. Fischer also realized the importance of correlating the configurations of amino acids with those of hydroxy acids and made some efforts towards that end but soon realized that the usual chemical methods were inadequate for that purpose. This problem received considerable attention in other laboratories and substantial

progress towards its solution has been made in recent years.⁻ This review will be devoted principally to these achievements. A⁻ brief review, however, will be given of the work on the correlation of the configurations of hydroxy acids with those of the sugars and of the mutual relationships of the configuration of individual amino acids.

The work on the two last-mentioned problems was accomplished by purely chemical methods. The chemical reactions employed were such that the asymmetric carbon atom was not involved.

Correlation of Hydroxy Acids with Sugars.

The first effort of a comprehensive treatment of this subject is that by Fischer. Prior to Fischer, isolated attempts were made in this direction. The first important contribution by Fischer was the one which established the configuration of d-tartaric acid, which was prepared by the degradation of l-rhamnose through the following steps:

$$
\begin{array}{ccc}
\underset{\text{I}}{\left[\begin{array}{c} \text{H} \quad \text{OH} \\ \diagdown \diagup \\ \text{C} \\ | \\ \text{HCOH} \\ | \\ \text{HCOH} \\ | \\ \text{CH} \\ | \\ \text{OHCH} \\ | \\ \text{CH}_3 \end{array}\right]}\text{O}
&
\begin{array}{c} \text{by the process} \\ \text{of Wohl} \\ \longrightarrow \end{array}
&
\underset{\text{II}}{\left[\begin{array}{c} \text{H} \quad \text{OH} \\ \diagdown \diagup \\ \text{C} \\ | \\ \text{HCOH} \\ | \\ \text{OHCH} \\ | \\ \text{CH} \\ | \\ \text{CH}_3 \end{array}\right]}\text{O}
&
\begin{array}{c} \text{by nitric acid} \\ \text{oxidation} \\ \longrightarrow \end{array}
\end{array}
$$

$$
\begin{array}{c}
\text{COOH} \\ | \\ \text{HCOH} \\ | \\ \text{OHCH} \\ | \\ \text{COOH}
\end{array}
$$

dextro-tartaric acid.

III
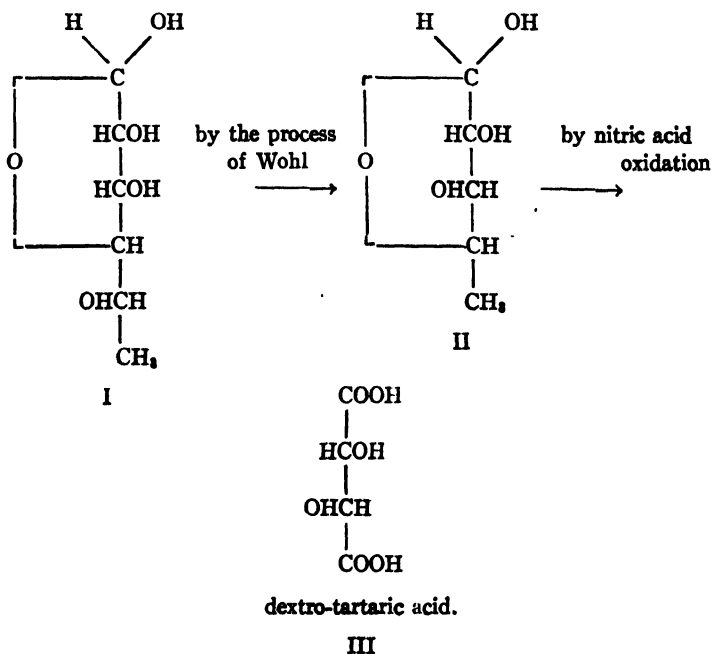
In 1875 Bremer converted by the action of hydroiodic acid dextro-tartaric into dextro-malic acid and in this he saw conclusive evi-

dence of the similarity of the allocation of the hydroxyl in dextro-malic and dextro-tartaric acids.

Prior to the time of Fischer's work, it had been shown that dextro-malic acid could be obtained from levo-aspartic acid. Fischer, in the early days of his work, thought that these three independent observations justified the conclusion that dextro-tartaric, dextro-malic and levo-aspartic acids are configurationally related.

But in 1893, the phenomenon of Walden Inversion was discovered and thereupon Fischer, in 1896, qualified the conclusions regarding the configuration of the four substances by a proviso that a Walden Inversion had not taken place in the course of the reactions which led from one to the other. As it happened, subsequent and more reliable methods which excluded the possibility of Walden Inversion established the same relationships which were formulated by Bremer.

The following figures, IV, V, VI, VII, VIII illustrate the set of reactions by which dextro-tartaric acid was converted into dextro-malic acid by K. Freudenberg and F. Brauns.

$$
\begin{array}{ccccccccc}
\text{COOH} & & \text{COOCH}_3 & & \text{COOCH}_3 & & \text{COOH} & & \text{COOH} \\
| & & | & & | & & | & & | \\
\text{HCOH} & & \text{HCOCOCH}_3 & & \text{HCOCOCH}_3 & & \text{HCOH} & & \text{HCOH} \\
| & \rightarrow & | & \rightarrow & | & \rightarrow & | & \rightarrow & | \\
\text{OHCH} & & \text{OHCH} & & \text{CHCl} & & \text{CHCl} & & \text{CH}_2 \\
| & & | & & | & & | & & | \\
\text{COOH} & & \text{COOCH}_3 & & \text{COOCH}_3 & & \text{COOH} & & \text{COOH} \\
\end{array}
$$

dextro.   dextro.

IV   V   VI   VII   VIII
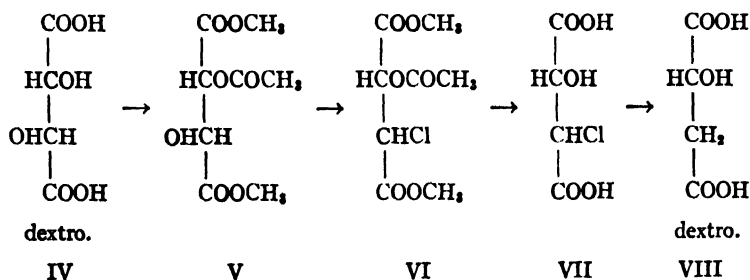
In an investigation which preceded the one just mentioned, K. Freudenberg correlated the configurations of malic, glyceric and lactic acids by the following set of reactions.

$$
\begin{array}{ccccccccc}
\text{COOH} & & \text{CONH}_2 & & \text{COOH} & & \text{COOH} & & \text{COOH} \\
| & & | & & | & & | & & | \\
\text{OHCH} & & \text{OHCH} & & \text{OHCH} & & \text{OHCH} & & \text{OHCH} \\
| & \rightarrow & | & \rightarrow & | & \rightarrow & | & \rightarrow & | & \rightarrow \\
\text{CH}_2 & & \text{CH}_2 & & \text{CH}_2 & & \text{CH}_2\text{NH}_2 & & \text{CH}_2\text{OH} \\
| & & | & & | & & & & \\
\text{COOH} & & \text{CONH}_2 & & \text{CONH}_2 & & & & \\
\end{array}
$$

natural,   malamid.   malaminic   levo-   dextro-glyceric
levo.   acid.   isoserine.   acid.

IX   X   XI   XII   XIII

$$\begin{array}{ccc}
\text{COOH} & & \text{COOH} \\
| & & | \\
\text{OHCH} & \rightarrow & \text{OHCH} \\
| & & | \\
\text{CH}_2\text{Br} & & \text{CH}_3
\end{array}$$

dextro-3-　　dextro-
bromo-　　lactic
lactic acid.　　acid.

XIV　　XV
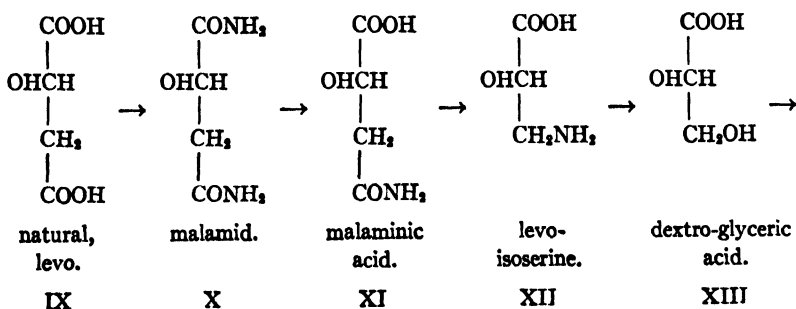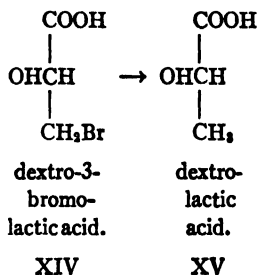
In a very ingenious and laborious way, Wohl and his co-workers correlated on the one hand, the configuration of glyceric aldehyde with tartaric acid and on the other, that of glyceric aldehyde with glyceric acid. Thus, in coöperation with Momber, he established the relationship between dextro-glyceric aldehyde and levo-tartaric acid in the following way:

$$\begin{array}{ccc}
\text{CHO} & & \text{COOH} \\
| & \text{by addition} & | \\
\text{HC—OH} & \text{of prussic} & \text{OHCH} \\
| & \text{acid} \quad\longrightarrow & | \\
\text{CH}_2\text{OH} & & \text{HCOH} \\
& & | \\
& & \text{CH}_2\text{OH}
\end{array}$$

dextro-　　　　levo-
glyceric　　　　tartaric
aldehyde.　　　acid.

XVI　　　　　XVII
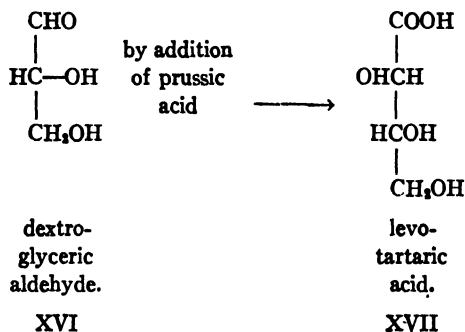
With R. Schellenberg he oxidized glyceric aldehyde to the corresponding acid,

$$\begin{array}{ccc}
\text{CHO} & & \text{COOH} \\
| & & | \\
\text{HCOH} & \longrightarrow & \text{HCOH} \\
| & & | \\
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH}
\end{array}$$

dextro-　　　levo-
glyceric　　　glyceric
aldehyde.　　acid.

. XVIII
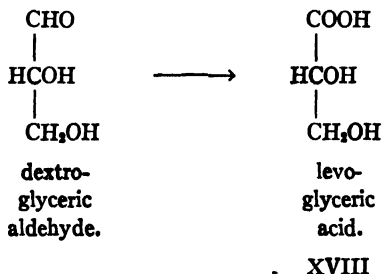
Thus, on the basis of all this very ingenious and technically difficult work the following relationships are established. Carbon atoms 3 and 4 of glucose are taken as points of reference.

CHO
|
CHOH
|
OHCH   COOH
| |
HCOH   OHCH   (as carbon 4   CHO   (as carbon 4
| | of d-glucose) | of d-glucose)
HCOH   ⟶   HCOH   ⟶   HCOH   ⟶
| | |
CH₂OH   COOH   CH₂OH

d-glucose.   levo-tartaric acid.   dextro-glyceric
                                                      aldehyde.

COOH   COOH   COOH
| | |
ГCOH   (as carbon 4   HCOH   (as carbon 4   HCOH
| of d-glucose) | of d-glucose) |
CH₂OH   ⟶   CH₂   ⟶   CH₃
                            |
                            COOH

levo-glyceric acid.   dextro-malic acid.   levo-lactic
                                                        acid.
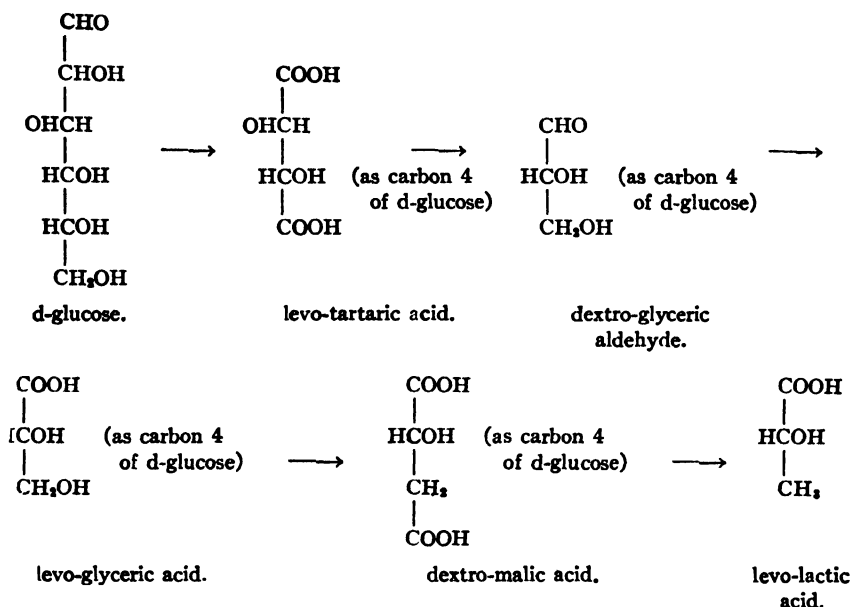
XIX

The dextro and levo, refer only to the direction of rotation whereas the letter *d*, as in *d-glucose*, indicates that the sugar belongs to the d series. Rosanoff's classification of the d and l series should be generally accepted. On the basis of this classification, dextro-tartaric, dextro-malic, levo-glyceric and levo-lactic belong to the d series. The designation of d and l series is a matter of convention; the relationship given in figure XIX are facts and correlate the other substances with d-glucose, if carbon atom (2) of d-glucose is taken as point of reference.

Stereochemical Relationships of 2-Amino Acids.

The pioneer work in this direction was also done by E. Fischer. In coöperation with K. Raske, he converted levo-serine into levo-l-amino-2-chloropropionic acid and this into dextro-alanine and later, also in coöperation with Raske, he converted levo-l-amino-

2-chloropropionic acid into levo-cystine. The reactions involved are presented in the following figures:

COOH
|
NH₂CH
|
CH₃

```
        COOH                    COOH          dextro-alanine.
         |                       |
       NH₂CH        ⟶         NH₂CH
         |                       |                    COOH
       CH₂OH                   CH₂Cl                   |
                                                     NH₂CH
                                                       |
                                                     CH₂SH

      levo-serine.          levo-1-amino-2-        levo-cystine.
                            chloropropionic
                                 acid.
```

$$COOH \\ | \\ NH_2CH \\ | \\ CH_3$$

levo-serine.   levo-1-amino-2-chloropropionic acid.   dextro-alanine.   levo-cystine.

XX
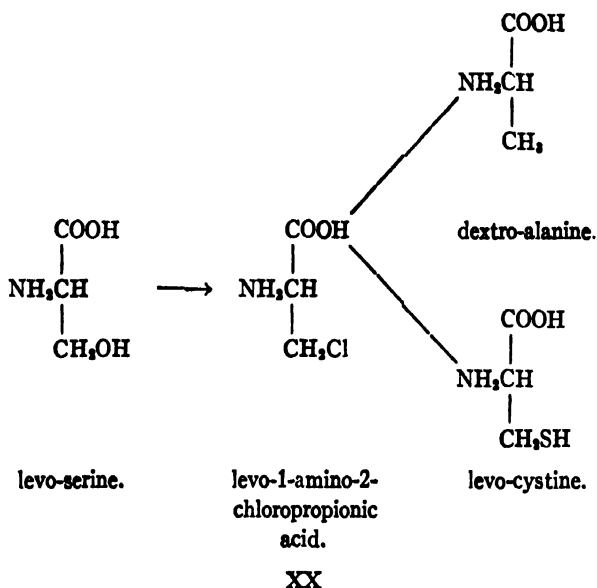
It is evident that all these amino acids belong to one and the same series although they rotate in opposite directions. More recently, Karrer in coöperation with Schlosser converted levo-asparagine into dextro-diamino-propionic acid through the following set of reactions.

```
   CONH₂                CH₂NH               CH₂NH₂
    |                    |    ╲               |
   CH₂        ⟶         |     CO    ⟶       CHNH₂
    |                    |    ╱               |
  CHNHCOCH₃             CHNH                 COOH
    |                    |
   COOH                 COOH

 levo-asparagine.    levo-glyoxalidon-    dextro-diamino-
                     2-carbonic acid-5.   propionic acid.

     XXI                 XXII                XXIII
```
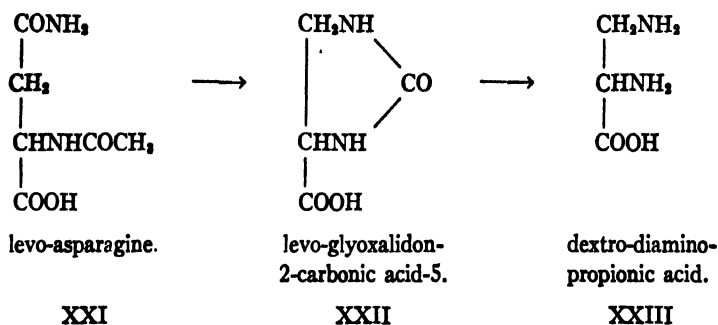
Later, Karrer converted dextro-serine into levo-diamino-propionic acid, thus having correlated levo-aspartic acid with levo-serine.

COOH   COOH
| |
NH$_2$CH ⟶ NH$_2$CH
| |
CH$_2$OH   CH$_2$NH$_2$

levo-serine.   dextro-diamino-
         propionic.
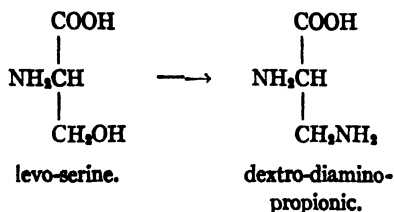
XXIV

Mention also should be made of the work of E. Waser and E. Brauchli, who advanced evidence in favor of configurational relationship between l-tyrosine and l-phenyl-alanine.

The latest contribution to the correlation of amino acids is the work of W. Langewhick from the laboratory of K. Freudenberg. This author converted histidine into benzoyl-levo-aspartic acid by the following set of reactions.

COOCH$_3$ COOCH$_3$ COOCH$_3$ COOH
| | | |
CHNH$_2$ CHNHCOC$_6$H$_5$ CHNHCOC$_6$H$_5$ C$_6$H$_5$COHN CH
| ⟶ | ⟶ | ⟶ |
CH$_2$ CH$_2$ through CH$_2$ CH$_2$
| | ozone. | |
C—NH   CNHCOC$_6$H$_5$ CONHCOC$_6$H$_5$ COOH
‖   ‖
| CH CH · NHCOC$_6$H$_5$
C—N

Hystidine   benzoyl-levo-
methyl ester.   aspartic acid.
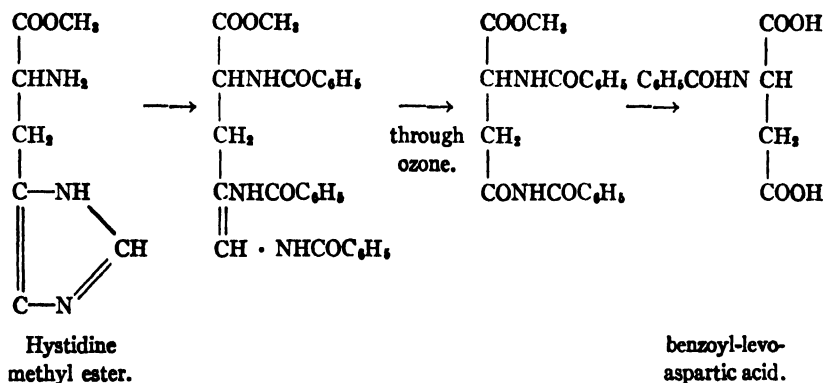
XXV

This concludes the list of amino acids of which the mutual stereochemical relationships have been established by chemical reactions which did not involve the asymmetric carbon atom.

While the work thus far reviewed was in progress, new problems came to the fore of which the solution at that time and still now seems unapproachable by the purely chemical method. Prominent among these were: The differentiation between dynamic isomers such as α- and β-sugars, ring isomers in the sugars, and above all, the problems which arise from the phenomenon of Walden Inversion.

For the solution of these new problems, recourse was taken to physical methods and the review which is to follow will be devoted to a survey of efforts in this direction. Several authors were engaged on these problems. The methods employed in each were in certain respects similar but not identical and the results obtained by different methods, in the majority of instances, were mutually corroborative. For clearness it may be advantageous to sketch separately the progress brought about by each individual method.

Conclusions Based on van't Hoff's Superposition Theory.

First Phase.

The pioneer work on the application of van't Hoff's theory for the purpose of bringing out configurational relationships was done by C. S. Hudson. A complete review of this work will not be attempted in this place for several reasons. It would occupy more space than the scope of the article permits. To some extent, it is being reviewed by Hudson himself. In this sketch only an outline of Hudson's method of reasoning is given.

The first paper by Hudson on this subject bears the title "The Significance of Certain Numerical Relations in the Sugar Group" and is concerned principally with the configuration of carbon atom (1) of the simple sugars and of their glycosides. The deductions made in that paper are based on two fundamental principles: one is that of van't Hoff, postulating that the molecular rotation of a substance with more than one asymmetric group is equal to the algebraic sum of the rotations of the individual groups. The second refers to the structure of simple sugars. The discovery of α- and β-isomers of simple sugars and of their glycosides led E. Fischer and other sugar chemists to accept for simple sugars the structure suggested by Tollens and now known as the oxidic structure, namely,

$$R\ CH \cdot CHOH \cdot CHOH \cdot C \overset{\Large H}{\underset{\Large OH}{\diagup}}$$

Here R stands either for H, $(C_nH_{2n}+_1)$ or for $CH_2OH(CHOH)_n$. Hudson pointed out that the molecular rotation of a simple sugar may be regarded as the algebraic sum of the rotations of carbon atom (1) and of that of the rest of the molecule. Thus, designating the α-isomer

the β-isomer will have to be expressed

Thus, the sum of the molecular rotation represents the value of 2A and the difference that of 2B.

By the use of this method, Hudson succeeded in establishing important relationships concerning the configuration of carbon atom (1) of simple sugars and of their glycosides; later, he extended the method for exploring the structure of several di- and trisaccharides as regards the α- or β-character of their glycosidic unions. The work has been both stimulating and important.

Second Phase.

This phase relates to stereochemical relationships of hydroxy acids. In search for a clue to the solution of the problem of the allocation of the amino group in 2-amino-hexonic acids, P. A. Levene was led to test whether the superposition theory of van't Hoff holds also for hexonic acids. The reasoning of Levene was analogous to that of Hudson. The molecular rotation of a hexonic acid may be regarded as the sum of rotations of two parts: one consisting of carbon atoms (1) and (2) and the other including carbon atoms (3), (4),

(5) and (6). Thus, taking a pair of epimeric hexonic acids, each isomer may be represented as follows:

$$
\underbrace{CH_2OH \cdot CHOH \cdot CHOH \cdot CHOH}_{A} \cdot \overset{\displaystyle H}{\underset{\displaystyle OH}{C}}\text{—COOH}
$$

$$
+ B
$$

$$
COOH\text{—}\overset{\displaystyle OH}{\underset{\displaystyle H}{C}} \cdot \underbrace{CHOH \cdot CHOH \cdot CHOH \cdot CH_2OH}_{A}
$$

$$
- B
$$
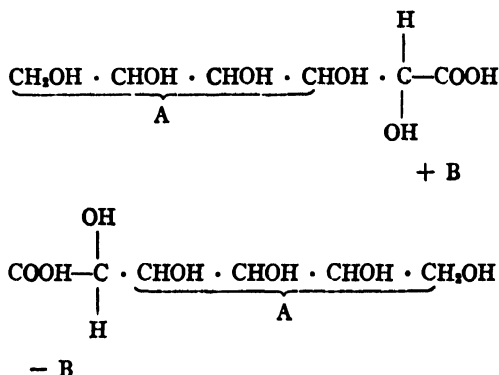
Here then the sum of the molecular rotations of the two acids is equal to 2A, and the difference to 2B.

Analysis of the data published by different writers but principally by Nef on the rotations of metal salts, alkaloidal salts and of phenyl-hydrazides of a number of sugar acids revealed the regularity in the direction of rotation of the carbon atoms (2) of the entire group of substances, namely, it was found that in all acids which had the allocation of the hydroxyl of carbon atom (2) as in the d-gluconic acid, the rotation of B was to the right and in those with the configuration of carbon (2) as in d-mannonic acid, the rotation of B was to the left. Later in coöperation with G. M. Meyer, Levene prepared a series of metal salts of sugar acids and later a series of phenyl-hydrazides. The original conclusions of Levene were substantiated by the more comprehensive analysis. Hudson also lent his attention to the optical behavior of sugar acids and noted that the rotation of B in the phenyl-hydrazides of sugar acids was quantitatively higher than that of A, and that therefore the direction of rotation of carbon atom (2) is in the same direction as that of the phenyl-hydrazide. Hence, according to Hudson, it was not necessary to possess a pair of epimers in order to determine the direction of rotation of carbon atom (2). Later Weerman and also Hudson and his co-workers directed their attention to the amides of sugar acids. Hudson further extended his observations to acids of simpler

structure, namely, to tartaric and malic, the configurations of which were established by chemical means and he found that also in the case of these acids the rule held, namely, d-tartaric acid amide

$$
\begin{array}{c}
\text{OH} \quad \text{H} \\
\text{CONH}_2 - \text{C} - \text{C} - \text{CONH}_2 \\
\text{H} \quad \text{OH}
\end{array}
$$
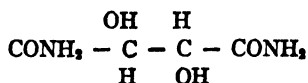
in agreement with the prediction rotated to the right and l-malic

$$
\begin{array}{c}
\text{OH} \\
\text{CONH}_2 - \text{CH}_2 - \text{C} - \text{CONH}_2 \\
\text{H}
\end{array}
$$
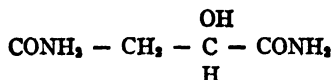
to the left. Hudson then applied the rule to levo-mandelic acid and attributed to it the configuration of the l-acids. Freudenberg, however, later modified the rule of Hudson to read that all such acids are to be designated as d-acids of which the amides have a higher dextro-rotation than the free acids. When so formulated, the rule held for all acids of known configuration and also for mandelic acid.

The case of levo-mandelic acid is important for the following reason. Mandelic acid is levo-rotatory $(-153°)$ and forms a levo-rotatory amide $(-95.5°)$ and hence, according to the rule of Hudson, should be regarded as an l-acid. On the other hand, hexahydromandelic acid obtained by the hydrogenation of levo-mandelic acid is levo-rotatory $(-26.6°)$ and forms a dextro-rotatory amide. Thus, according to the original rule of Hudson, different configurations should be assigned to the two acids but according to the rule as modified by Freudenberg, the same configuration of d-acids is assigned to both.

Thus, in regard to the configuration of hydroxy acids, the work of Levene, Hudson, Weerman and Freudenberg led to the formulation of definite rules which permit the determination of the configuration of a hydroxy acid on the basis of its optical behavior. It must be emphasized, however, that all the work thus far reviewed was concerned principally with developing a physical method for ascertaining configurational relationships of hydroxy derivatives only. The next task was to discover a way for coördinating configurations of amino and hydroxy derivatives. The work of Levene on hexo-

saminic acids had that aim. Similar was the aim of the work of Clough. The methods employed by Clough were the outgrowth of much important work on the relationship of chemical structure and optical rotation which was done by many very prominent chemists, namely, Frankland, Armstrong, McKenzie, Patterson, Pickard and Kenyon and others. The principal contributions bearing on the topic under discussion are reviewed in the following section.

Influence of External Conditions Such as Temperature and Character of Solvent and of Substitution on Optical Rotation.

It may be well to begin the survey of the work on this topic by reference to the publication by Frankland and Wharton in 1896. The investigation was concerned with the influence of chemical structure on the optical activity. In that, the rotatory powers of dibenzyol and ditoluyltartrates are discussed. The unexpected observation made in the course of the work is contained in the following quotation: "This is a very remarkable series of rotations exhibiting, as it does, a phenomenon which, as far as we are aware, has not hitherto been observed, namely, the passage through a maximum in the change of rotation, brought about by change of temperature, or, in other words, a change in the sign of the sensitiveness of the rotation due to the temperature."

The results are given in table 1.

In a more extensive way the influence of solvent and of temperature on rotation was investigated by T. S. Patterson. At the outset of his work, Patterson was engaged in searching for the causes of the variation of the rotatory power of a substance in different solvents. The method employed by this author consisted in comparing the curves expressing the changes of rotations with respect to changes of temperature. Patterson discarded the then-prevailing theory that the cause of the variation of rotation was due to the differences in the state of aggregation in different solvents and instead was inclined to accept the view that the principle variable was the molecular solution volume. Soon, however, the work assumed a broader aspect, namely, the search for such peculiarities or singular points in the temperature rotation (T.R.) curves which could offer a

basis of comparison between structurally related substances. Pat-
terson was impressed by the fact first noted by Frankland and
Wharton that every T.R. curve had a sinuous form exhibiting a
maximum and a minimum. This author then concentrated his
attention on the position of the maximum point with the change of
concentration and of temperature. Figure XXVII presents the
results of measurements of the molecular rotation of aqueous solu-

TABLE 1.

Rotation of Ethylic Dibenzoyltartrate.

(Length of polarimeter tube in each case was 44 mm.)

At $\quad 1.3°\ [\alpha]_D = \dfrac{-30.06°}{0.44 \times 1.2121} = -56.36°;\ [\delta]_D = -477.5.$

At $\ 18.0°\ [\alpha]_D = \dfrac{-31.29°}{0.44 \times 1.1979} = -59.36°;\ [\delta]_D = -499.0.$

At $\ 38.0°\ [\alpha]_D = \dfrac{-32.06°}{0.44 \times 1.1809} = -61.70°;\ [\delta]_D = -514.0.$

At $\ 44.0°\ [\alpha]_D = \dfrac{-32.10°}{0.44 \times 1.1758} = -62.05°;\ [\delta]_D = -515.0.$

At $\ 53.5°\ [\alpha]_D = \dfrac{-32.00°}{0.44 \times 1.1677} = -62.28°;\ [\delta]_D = -514.5.$

At $\ 60.0°\ [\alpha]_D = \dfrac{-31.85°}{0.44 \times 1.1622} = -62.28°;\ [\delta]_D = -513.0.$

At $\ 77.5°\ [\alpha]_D = \dfrac{-31.37°}{0.44 \times 1.1472} = -62.15°;\ [\delta]_D = -507.0.$

At $100.0°\ [\alpha]_D = \dfrac{-30.16°}{0.44 \times 1.1280} = -60.77°;\ [\delta]_D = -490.5.$

At $109.5°\ [\alpha]_D = \dfrac{-29.51°}{0.44 \times 1.1199} = -59.89°;\ [\delta]_D = -481.0.$

At $136.5°\ [\alpha]_D = \dfrac{-27.38°}{0.44 \times 1.0970} = -56.72°;\ [\delta]_D = -450.0.$

At $182.5°\ [\alpha]_D = \dfrac{-24.03°}{0.44 \times 1.0571} = -51.66°;\ [\delta]_D = -399.5.$

tions of potassium ethyltartrate. In these experiments the position
of the maximum varied with concentration of the solute. It varied
also with the solvent and with the character of the chemical group
introduced into the tartaric acid.

Patterson, after work which extended over many years and which
embraces a great number of substances, came to the conclusion that

closely related chemical substances such as esters of tartaric acid
exhibit similar temperature rotation curves, although the maximum
rotation may occur at different temperatures and at different rotations.
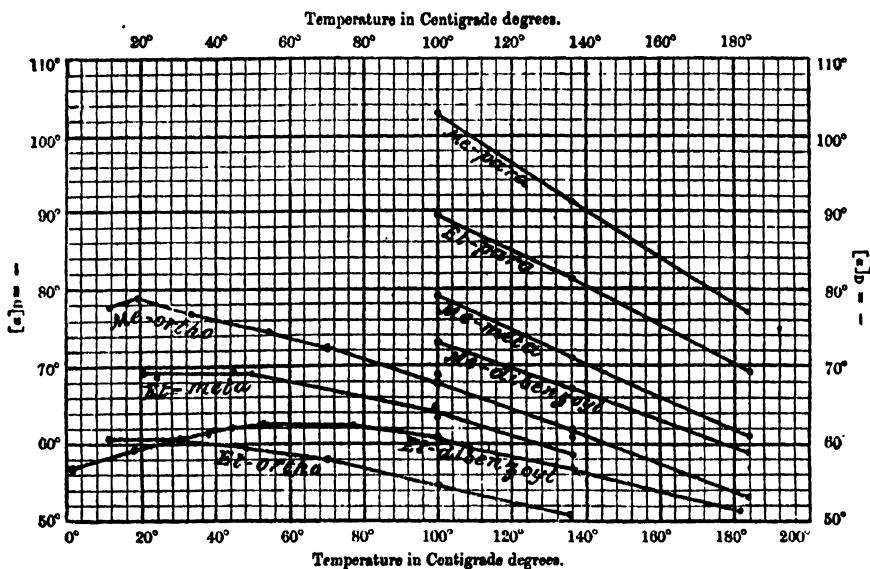
FIG. XXVI. INFLUENCE OF TEMPERATURE ON THE SPECIFIC ROTATION OF
METHYLIC AND ETHYLIC DITOLUYLTARTRATES, AND OF METHYLIC AND
ETHYLIC DIBENZOYLTARTRATES
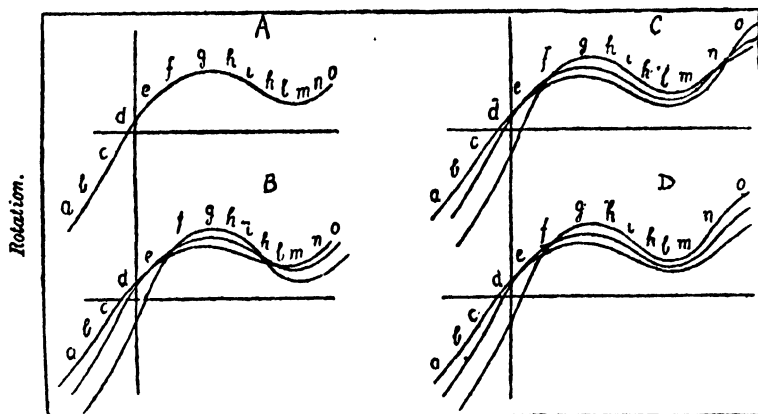
FIG. XXVII.

The significance of the temperature rotation curve as a basis of comparison of configurationally similar substances was further demonstrated by Pickard and Kenyon in a long series of investigations on the derivatives of simple secondary carbinols.

As experience and data accumulated, it became evident that another important factor had to be considered in comparing configurationally related substances, namely, the color of the light employed for the measurement of the rotation. In this connection a very important discovery was made by Armstrong and Walker. These authors suggest that the specific rotations of substances for light of different refrangibilities can be arranged diagrammatically and, what they term a "characteristic diagram" is formed by configurationally related substances. Armstrong and Walker describe the construction of the diagram in the following words:

> To construct the diagram characteristic of a substance, a reference line is drawn with a slope of unity and on this are plotted the various specific rotations of light of any of the refrangibilities observed. The points for other refrangibilities are then plotted on the ordinate passing through the points previously located on the reference line. The observations may be those made either in different solvents or at different concentrations.

Figure XXVIII represents the characteristic diagram for d-fructose in different concentrations. The measurements were made by Armstrong and Walker. In Figure XXIX specific rotations are plotted as functions of solvent.

The application of rotatory dispersion was further advanced by the very important work of T. M. Lowry who demonstrated that for the majority of organic substances, the rotatory dispersion can be expressed by Drude's formula

$$\alpha = \frac{K}{\lambda^2 - \lambda^2_0} \tag{I}$$

He termed the form of dispersion which can be expressed by this formula, "simple dispersion," in distinction from "complex dispersion" which could not be expressed by the simple formula and which re-

quired for its expression the two membered formula containing four constants:

$$\alpha = \frac{K_1}{\lambda^2 - \lambda^2_1} - \frac{K_2}{\lambda^2 - \lambda^2_2} \tag{II}$$

It is evident that K, in all cases where formula (I) holds for lights of all refrangibilities, is independent of the wave length, and hence
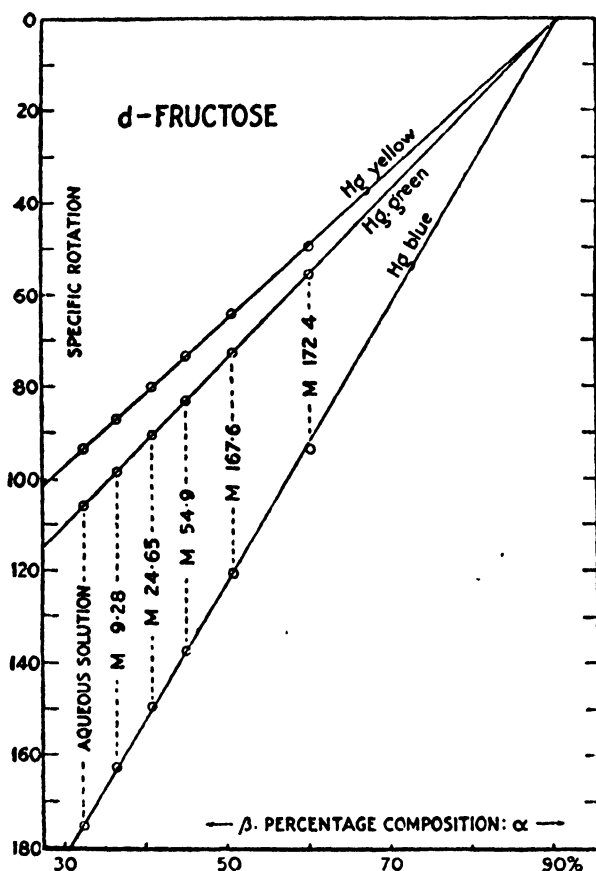
FIG. XXVIII. CHARACTERISTIC DIAGRAM OF d-FRUCTOSE

Equilibrium between α aud β forms altered by the addition of alcohol to the aqueous solution. Reference color, Hg green.

K — the rotation constant, expresses better the rotatory properties of a substance than the value of the specific rotation at one given wave length.
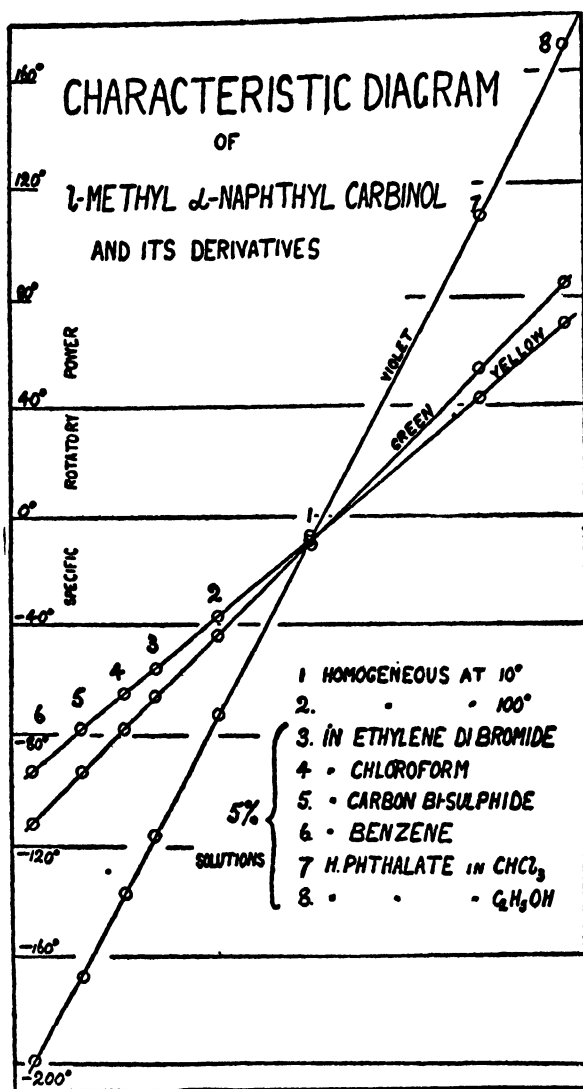
FIG. XXIX. CHARACTERISTIC DIAGRAM FOR l-METHYL-α-NAPHTHYL CARBINOL
AND ITS DERIVATIVES
The measurements were made by Pickard and Kenyon.

Very useful information was obtained when rotatory dispersion was measured at varying temperatures. Namely, it was observed that certain substances exhibited complex dispersion up to a certain

range of temperature changes and then followed the simple law. This indicates that the complexity of the solution is smaller at the temperatures exhibiting the simple dispersion and hence comparisons made at those temperatures are more serviceable.

To sum up, the studies in temperature rotation changes and those in rotatory dispersion, aimed at a method of expressing those rotatory powers of a substance which were, as far as possible, free from external influences. The methods here reviewed were particularly useful in correlating the configurational relationships of carbinols but were also applied to work which constitutes the main theme of this review and to which the following two sections are devoted.

Configurational Correlation between Amino Acids and Hydroxy Acids.

Chronologically, after the discovery of Walden Inversion, the systematic effort of correlating the configuration of amino acids with hydroxy acids was made by Levene, but the publication of Clough had a more comprehensive scope and for this reason, the discussion will begin with a review of his article, published in 1918. The principles developed by Frankland, Patterson, Pickard and Kenyon were accepted by Clough and made the basis for a comparison of the configurational relationships of hydroxy, amino and halogen acids.

Hydroxy Acid.

Clough began his discussion ·by the analysis of the influences of external conditions such as temperature, solvent and certain substituents on a series of acids of known configuration, namely, on levo-lactic, levo-glyceric, dextro-malic and dextro-tartaric. These acids, as developed in the earlier part of this review, are configurationally identical and because of their relationship to d-glucose may be designated d-acids.

Clough then extended his analysis to hydroxy acids of which the configuration had not been known. Thus he examined the influence of sodium chloride (or bromide) on dextro-β-phenyl-lactic acid and its methyl ester. On the basis of similarity of the action of these salts on this acid and on the derivatives of levo-malic, levo-lactic and dextro-tartaric acids, he assigned to dextro-β-phenyl-

lactic acid the configuration of the d-series. Of the many tables relating to the hydroxy acids the two pertaining to this case are given by way of an illustration of Clough's procedure.

TABLE 2.

The Influence of Sodium Bromide on the Optical Rotatory Power of Levo-Malic Acid, Dextro-Tartaric Acid, Methyl-Levo-Lactate, Methyl-Levo-Malate, and Methyl-Dextro-Tartrate in Methyl Alcoholic Solution.

COMPOUND	$[\alpha]_{gr.}^{15}$ (IN METHYL ALCOHOL C = 10)	$[\alpha]_{gr.}^{16}$ (IN METHYL ALCOHOLIC SODIUM BROMIDE N. C = 10)
Levo-malic acid...........................	−5.9°	+21.8°
Dextro-tartaric acid.......................	+2.6	−9.0
Methyl-levo-lactate.......................	+5.4	−5.8
Methyl-levo-malate.......................	−9.4	+9.1
Methyl-dextro-tartrate....................	+2.7	−12.6

TABLE 3.

The Influence of Sodium Haloids on the Optical Rotatory Powers of Dextro-β-Phenyl-Lactic Acid and Methyl-Dextro-β-Phenyl-Lactate in Solution.

COMPONENT	SOLVENT	$\frac{\Lambda}{C}$	$t°$	$\alpha gr._{(l=2)}^{t}$	$[\alpha]_{gr.}^{t}$
Dextro-β-phenyl-lactic acid...............	Water	2.505	20°	+1.28°	+25.5°
	Aqueous sodium chloride (4N)	0.665	20	+0.18	+13.5
	Methyl alcohol	10.0	20	+4.20	+21.0
	Methyl alcoholic sodium bromide (N)	10.0	20	−0.41	−2.0
Methyl-dextro-β-phenyl-lactate..............	Methyl alcohol	10.0	18	−0.90	−4.5
	Methyl alcoholic sodium bromide	10.0	18	−4.46	−22.3

In a similar way the behavior of levo-α-hydroxyisohexoic, levo-α-hydroxyglutaric and levo-α-hydroxyisovaleric acids was analyzed.

The conclusions regarding the configurations of hydroxy acids suggested by Clough are: "levo-lactic acid, levo-glyceric acid, dextro-malic acid, dextro-α-hydroxy butyric acid, dextro-α-hydroxyisovaleric

acid, dextro-α-hydroxyisohexonic acid, dextro-α-hydroxyglutaric acid and dextro-β-phenyl-lactic acid all possess the same relative configuration as dextro-tartaric acid."

2-Amino Acids.

Clough then proceeded to investigate the influence of inorganic salts and of different solvents on the optical rotatory power of amino acids.

The following tables contain the data of such information as was obtained by Clough or as collected by him from literature.

Applying to the analysis of the optical behavior of amino acids the same mode of reasoning as was used in connection with the hydroxy acids, Clough formulated the following conclusion regarding the configuration of naturally occurring amino acids.

Naturally occurring amino acids, commonly denoted as dextro-alanine, levo-serine, levo-aspartic acid, dextro-valine, levo-leucine, dextro-isoleucine, dextro-aminobutyric acid, dextro-glutamic acid, levo-phenyl-alanine, and levo-tyrosine all possess the same configuration. The configuration of these compounds has been denoted by the symbol "l."[1]

In a similar manner, Clough has analyzed the behavior of α-halogen acids. In connection with this class of compounds, the available data were very meagre. Nevertheless, Clough felt justified in drawing the following conclusion:

The dextro-rotatory α-halogen acids which have been isolated are assumed to be configurationally similar compounds. A comparison of the rotatory powers of the optically active α-bromoacylamino acids with those of the α-amino-acylamino acids leads to the view that the halogen acids mentioned above are configurationally related "l" amino-acids.

Thus, according to Clough, the naturally occurring amino acids have the configurations of l-tartaric acid and the dextro-halogen acids have the same configurations. Clough, however, states that

---

[1] Clough denotes by "d" such acids that have the configuration of d-tartaric acid as "l" those having the configuration of levo-tartaric acid. The present writer suggests the indication of the direction of rotation by the words "dextro," or "levo," and those having the configuration of d-tartaric acid as d-acids. This practice would be in harmony with the designations now in use in the sugar acids.

TABLE 4.

The Influence of Inorganic Salts on the Optical Rotatory Power of d-Glutamic Acid in Aqueous Solution.

SOLVENT	p	d^{25}	$\alpha_{gr.}^{25}$ ($l = 4$)	$[\alpha]_{gr.}^{25}$
Water............................	1.50	1.003	$+0.80°$	$+13.3°$
Aqueous sodium chloride (N)........	1.50	1.043	0.90	14.4
Aqueous sodium chloride ($4N$).......	1.51	1.152	1.10	15.8
Aqueous potassium chloride (N)......	1.53	1.050	0.95	14.8
Aqueous barium chloride (N)........	1.53	1.092	0.96	14.4
Aqueous barium bromide ($4N$).......	1.51	1.500	1.64	18.1
Aqueous sodium hydroxide (1 mol.)....	12.25	1.075	-1.88	-3.6
Aqueous sodium hydroxide (2 mols.)...	6.55	1.050	$+3.22$	$+11.7$
Aqueous hydrogen chloride (1.5 mols.)..	8.75			$+37.4$

TABLE 5.

The Influence of Solvents on the Optical Rotatory Power of Ethyl l-Aspartate.

SOLVENT	c	$\alpha_{gr.}^{18}$ (l = 2)	$[\alpha]_{gr.}^{18}$
None	10.92°	−6.34° (l = 0.5)	−11.7°
Benzene	11.02	−2.75	−12.6
Chloroform	20.0	−2.13	−9.7
Acetone*	21.7	−3.50	−8.8
Water	12.25	+1.83	+4.2
Water	10.81	1.03	4.2
Aqueous sodium chloride (4N)	14.50	1.44	6.7
Aqueous barium bromide (4N)	13.21	3.34	11.5
Aqueous calcium chloride (5N)	11.37	3.84	14.5
Aqueous hydrogen chloride (N)		2.73	12.0
Methyl alcohol	20.0	$[\alpha]_{gr.}^{20}$ −0.19	$[\alpha]_{gr.}^{20}$ −0.5
Methyl alcoholic sodium bromide (N)	20.0	$[\alpha]_{gr.}^{20}$ +1.72	$[\alpha]_{gr.}^{20}$ +4.3

* Ethyl l-aspartate reacts with acetone, the rotation changing from $\alpha_{gr.}$ −3.50° to $\alpha_{gr.}$ −56.6° (c = 20) in fifteen hours.

TABLE 6.

The Optical Rotatory Powers ($[\alpha]^{26}_D$) of α-Amino Acids and of Certain Derivatives from Them.

AMINO ACID	WATER	HYDRO-CHLORIC ACID	BENZOYLAMINO-ACID (IN KOH, AQ)	AMINO-ACYLGLYCINE (IN WATER)	GLYCYLAMINO-ACID (IN WATER)	HYDANTOIN*
d-Alanine[1]	+2.7°	+14.3°	+36.5°	+50.2°	−50.0°	+50.6° (water)
l-Serine[2]	−6.8	+14.4	+43.6 (in NaOH, aq.) (p-nitrobenzoyl)	−	−	−
l-β-Chloro-α-amino-propionic acid[3]	−15.5	+0.9	−	−	−	−
d-α-Amino-butyric acid[4]	+8.0	+19.6	+30.7 (in NaOH, aq.)	+86.4 (K) +26.8 (A & C)	−20.3	−
d-Valine[5]	+6.4	+28.7	−	+90	−19.7	−97.5 (phenyl-hydantoin in alcohol)
d-α-Amino-hexoic acid[6]	−	+23.0	+21.9	−	−	−
l-Leucine[7]	−10.3	+15.6	+6.6 (in NaOH, aq.)	+85.5	−35.0	−68.2 (in NaOH, aq.)

* Hydantoins, Dakin and Dudley, J. Biol. Chem., 1913, 17, 29; 1914, 18, 48; T., 1915, 107, 434.

1 Fischer, Ber., 1905, 38, 2914; 1906, 39, 453; 1907, 40, 943.

2 Fischer and Jacobs, Ber., 1906, 39, 2942; Fischer and Raske, Ber., 1907, 40, 3717.

3 Fischer and Raske, loc. cit.

4 Fischer and Mouneyrat, Ber., 1900, 33, 2383; Koelker, Zeitsch. physiol. Chem., 1911, 73, 312; Abderhalden and Chang, ibid., 1912, 77, 471.

5 Fischer, Ber., 1906, 29, 2320; Fischer and Scheibler, Annalen, 1908, 363, 136.

6 Fischer and Hagenbach, Ber., 1901, 34, 3764.

7 Fischer, Ber., 1900, 33, 2370; Ber., 1906, 39, 2893; Fischer and Steingroever, Annalen, 1909, 365, 167.

TABLE 7.

The Optical Rotatory Powers ($[\alpha]_D^{20}$) of α-Amino Acids and of Certain Derivatives from Them.

AMINO ACID	WATER	HYDRO-CHLORIC ACID	BENZOYLAMINO-ACID (IN KOH, AQ.)	AMINO-ACYLGLY-CINE (IN WATER)	GLYCYLAM-INO-ACID (IN WATER)	HYDANTOIN[*]
d-Isoleucine[8].........	+11.3°	+40.6°	+26.4° (in NaOH, aq.)	+33.6°	-14.7°	—
l-Asparagine[9].........	-4.9	+28.5	—	—	-6.4	—
l-Aspartic acid[10].........	+4.3	+25.7	+37.4	—	+11.1	-125 (in NaOH, aq.)
d-Glutamic acid[11].........	+9.9	+30.8	+18.7	—	-6.3	-79 (in NaOH, aq.)
l-Phenyl-alanine[12].........	-35.3	-7.1	+17.1	+54.2	+41.4	-96.4 (in 50 per cent alcohol)
l-Tyrosine[13].........	—	-8.6	+19.2	—	—	-143 (in NaOH, aq.)

* Hydantoins, Dakin and Dudley, J. Biol. Chem., 1913, 17, 29; 1914, 18, 48; T., 1915, 107, 434.

8 Locquin, Bull. Soc. chim., 1907 [iv], 1, 595; Abderhalden, Hirsch, and Schuler, Ber., 1909, 42, 3394.

9 Fischer and Koenigs, Ber., 1904, 37, 4585.

10 Fischer, Ber., 1899, 32, 2451; Fischer and Fiedler, Annalen, 1910, 375, 181.

11 Fischer, Krop, and Stahlschmidt, Annalen, 1909, 365, 189.

12 Fischer and Mouneyrat, loc. cit.; Fischer and Schoeller, Annalen, 1907, 357, 1.

13 Fischer, Ber., 1899, 32, 3638.

further investigations are desirable in order that a definite decision may be reached.

Simultaneously with Clough, Karrer, in coöperation with Kaase, measured the optical rotatory dispersion of dextro-glutamic acid, and of several of its derivatives, the ethyl ester, levo-chloroglutaric and dextro-hydroxyglutaric. The amino acid, the hydroxy acid and several other derivatives exhibited a rise in dextro-rotation with the decrease in the wave length; the chloroderivative exhibited an increase to the left. Karrer and Kaase drew the conclusion that the hydroxy and the amino acids examined by them were configurationally related, whereas the levo-chloro acid was enantiomorphously related to the former two. With respect to the amino and chloro-

TABLE 8.

	K_M NATURAL (DEXTRO) ALANINE	K_M DEXTRO-LACTIC ACID
1. Amide of the benzoyl derivative......................	$< +29$	$+36$
2. Ethyl ester benzoyl derivative........................	$+3$	$+10.5$
3. Methyl ester benzoyl derivative.......................	$0\ (\pm 1)$	$+ 7.5$
4. Ethyl ester of the hexahydrobenzoyl derivative..........	$-19\ (\pm 4)$	-15
5. Ethyl ester of the acetyl derivative....................	-22	-25
6. Amide of toluolsulfo derivative........................	$-26\ (\pm 8)$	$-28\ (\pm 3)$
7. Ethyl ester toluolsulfo derivative......................	-26	-39

acid, the conclusions of Karrer and Kaase coincided with that of Clough.

Very recently, Freudenberg and Rhino measured the rotation constant of several derivatives of dextro-alanine with similar derivatives of dextro-lactic acid. The calculations were made by the Akermann formula which is Drude's rotatory dispersion formula. Its applicability for the measurement of rotatory dispersion of organic compounds was first demonstrated by Lowry. Table 8 contains the results of the observations of Freudenberg and Rhino.

On the basis of these results, Freudenberg and Rhino consider dextro-alanine and dextro-lactic acids configurationally similar and denote the two acids l-acids.

Thus, practically every one of the methods introduced by Frankland, Patterson, Armstrong, Pickard and Kenyon for the study of stereochemical relationships was made use of in the investigations of configurational relationships between hydroxy acids, halogen acids and amino acids. Thus far, the conclusions have been mutually corroborative.

The Influence on the Optical Rotation of the Change of Polarity of One of the Groups Attached to the Asymmetric Carbon Atom Brought About without the Process of Substitution.

It was pointed out in the early section of this review that the correlations in the group of hydroxy acids were to a large extent established by methods of pure organic chemistry. Also those that have not yet been correlated by these methods undoubtedly will be some day in the not distant future. Many of the amino acids have been mutually correlated by purely chemical methods. It is not impossible that the halogen substituted acids will be mutually correlated by such methods. However, there is little hope that hydroxy, amino and halogen acids will be correlated by purely chemical methods. The cause of the difficulty lies in the phenomenon of Walden Inversion. When the condition under which the Inversion takes place is discovered, then the correlation between the configuration of the three groups of substances will become apparent of itself. The work on Walden Inversion done up to the present remains without practical value for the investigations into configurational relationships. Recently, Levene has undertaken a series of investigations which promises to throw some light on the circumstances under which the phenomenon of Walden Inversion occurs. The plan of the work consists in observing the changes in the optical activity of a substance which are brought about by changes in the polarity of one group attached to the asymmetric carbon atom when the change is accomplished without substitution. Levene, in cooperation with Mikeska, observed the changes in the optical behavior of secondary thioderivates and of the corresponding sulfonic acids. The results of the observations are the following. In the derivatives

of normal secondary alcohols, the changes in direction of rotation are as follows:

$$R_1 - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - R_2 \text{ by HI} \longrightarrow R_1 - \underset{\underset{X}{|}}{\overset{\overset{H}{|}}{C}} - R_2 \longrightarrow$$

dextro-rotatory.   Substitution,
                                 (X stands for
                                 halide) levo-
                                 rotatory.

$$R_1 - \underset{\underset{SH}{|}}{\overset{\overset{H}{|}}{C}} - R_2 \longrightarrow R_1 - \underset{\underset{\underset{CH}{|}}{SO_2}}{\overset{\overset{H}{|}}{C}} - R_2$$

Substitution   Change without
dextro-rotatory.   substitution
                                 levo-rotatory.
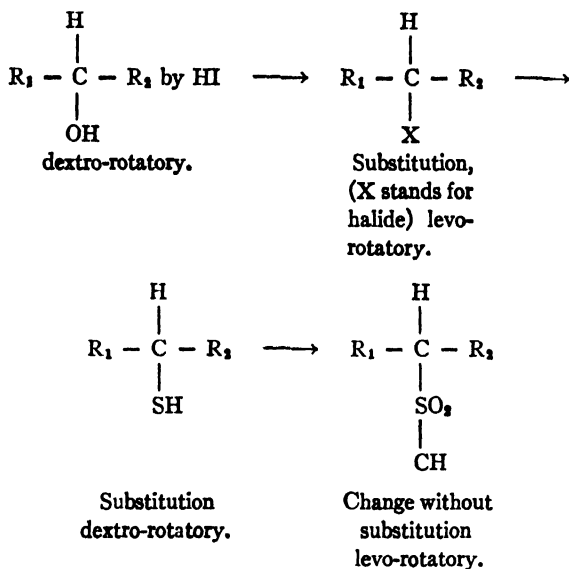
Thus, in this group of substances, the last change of the direction of rotation is brought about under conditions which exclude the possibility of a Walden Inversion and therefore, one is justified in concluding that normal aliphatic dextro-rotatory secondary alcohols are configurationally related to the levo-rotatory halides and that the above conversion of the alcohol into the halide has taken place without Walden Inversion.

Levene and Mikeska then extended their observations to α-substituted acids.

In this group of substances the change from the thio to the sulfonic acid derivative is not accompanied by the same change of rotation that was characteristic of the derivatives of the normal secondary alcohols. A decrease in dextro-rotation, perhaps, is exhibited by the sulfonic acid derivatives. This needs confirmation. The important fact, however, is that in the thio acid and in the sulfonic acid, there is observed the same character of change of rotation when the acid is converted into a metallic salt. Thus, dextro-thiolactic acid forms a levo-rotatory sodium salt, and the configurationally

related sulfopropionic acid shows a similar change of rotation when it is transformed into a metallic carboxylic salt. The results of the measurements are given in table 9.

At first glance the results summarized in this table seem confusing. Thus the dextro-rotatory thiolactic acid forms a levo-rotatory monobasic salt. It behaves in this respect similarly to dextro-lactic acid. The direction of rotation, however, is again changed when a second equivalent of sodium hydroxide is added to the solution. Thus the neutral salt is dextro-rotatory. In the sulfopropionic acid the direction of rotation is unaltered when the acid is converted into a monobasic salt, and is reversed in the dibasic salt. This apparently puz-

TABLE 9.

Optical Activity of the Salts of Thio and Sulfo Acids.

SUBSTANCE	FREE ACID $[M]_D$	MONO-SALT $[M]_D$	DI-SALT $[M]_D$	TRI-SALT $[M]_D$
l-Xanthopropionic acid................	$+107.68°$	$+45.05°$		
l-Thiolactic acid......................	$+58.98$	-5.58	$+7.32°$	
l-Sulfopropionic acid..................	$+13.76$	$+12.84$	-3.28	
l-Xanthosuccinic acid..................	$+95.05$	$+43.86$	$+8.65$	
l-Thiosuccinic acid....................	$+73.06$	$+41.58$	$+48.57$	$+41.09°$
l-Sulfosuccinic acid...................	$+44.25$	$+43.43$	$+29.22$	$+25.67$
d-Sulfobutane........................	-4.38	-6.29		

zling difference in the behavior of the two substances, however, can be readily explained. In dextro-thiolactic acid

$$CH_3 - \overset{\overset{\displaystyle SH}{|}}{\underset{\underset{\displaystyle H}{|}}{C}} - COOH$$
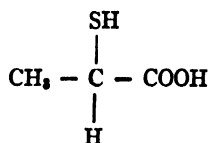
the carboxyl is the stronger acidic group. When the hydrogen of this group is replaced by a metal ion, the substance behaves as dextro-lactic acid, namely, the salt is levo-rotatory. When a second equivalent of the base is added, it replaces the hydrogen atom of the sulfide rest and then the direction of the rotation turns to the right.

In the sulfopropionic acid

$$CH_3 - \underset{\underset{H}{|}}{\overset{\overset{OH}{\underset{|}{SO_2}}}{C}} - COOH$$
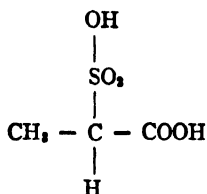
the stronger acidic group is the SO_2OH rest. The neutralization of this group brings about a slight change in the direction toward the left, as is the case when the thio group is neutralized in dextro-thiolactic acid. When a second equivalent of base is added, this forms a salt with the carboxyl group and then the direction of the rotation is shifted to the left as is the case when the carboxyl group is neutralized in the dextro-thiolactic or in the dextro-lactic acids.

In a similar way, the changes in rotations of the thio- and sulfosuccinic acids may be analyzed.

These observations are instructive inasmuch as the configurational relationships of the substances are known from the methods of their preparation and hence the observations show that substituted acids configurationally related exhibit similar changes of optical rotation when the acids are converted into the metallic salts even though the polarity of one of the groups attached to the asymmetric carbon atom is changed. Thus, dextro-lactic acid and dextro-alanine should also be regarded as configurationally related for the reason that in both, the free acid has a higher dextro-rotation than the metallic salt. The same conclusions regarding dextro-lactic acid and dextro-alanine were reached by Clough and by Freudenberg on other grounds. The evidence of Levene and Mikeska is more direct than that of the other writers.

The Stereochemical Relationships of Hexoses and Hexonic Acids to 2-Amino-Hexoses and 2-Amino-Hexonic Acids.

Work on this problem was undertaken prior to any other systematic investigation into the configurational relationship of amino and hydroxy derivatives. The original plan of the work was a comprehensive one. It was hoped first to correlate the simple sugars

and sugar acids with amino-hexoses and hexosaminic acids, then to reduce the latter to α-amino acids and thus to establish the configuration of amino acids by purely chemical methods. Work on amino-pentonic acids also was contemplated. Unfortunately, the attempts to reduce the 2-amino-hexonic acids to optically active 2-amino-caproic acids were not successful. Nevertheless, the work on hexosamines and on amino-hexonic acids furnished very important data for the elucidation of the configurational relationship of hydroxy and of amino acids.

The advantages which the sugar acids offer for the study of stereochemical relationships are the following: A pair of enantiomorphous substances differ from one another by only one property, the direction of the optical rotation. Two sugar acids enantiomorphous only with regard to carbon atom (2) differ, as a rule, in other physical properties in addition to the optical and often differ also in their chemical behavior. Furthermore, the acids can be reduced to the corresponding sugars, and these again differ markedly from one another. Thus, gluconic and mannonic acids are characterized not only by the optical behavior of the free acids and their salts, amides, and phenyl-hydrazides, but also by their solubilities, their tendencies to form crystalline lactones, etc., and in addition, by the physical and chemical properties of glucose and of mannose to which they can be reduced respectively.

Levene and his co-workers synthetically completed the series of 2-amino-hexonic acids (only one had been known prior to their work). Making use of the van't Hoff superposition theory in a manner similar to that applied by Hudson to sugars, they classified the eight d-2-amino-hexonic acids into two groups, namely,

Group I	*Group II*
Chitosaminic	Epichitosaminic
Chondrosaminic	Epichondrosaminic
Levo-xylohexosaminic	Dextro-xylohexosaminic
Levo-ribohexosaminic	Dextro-ribohexosaminic

The substances in each group are configurationally related in regard to carbon atom (2). The substances in group I and group II are enantiomorphously related in regard to carbon atom (2).

The basis for this classification is the comparison of the values of the optical rotations of carbon atom (2) in the hydrochlorides of the amino acids. The next problem was to establish the allocation of the amino groups in these acids. The configuration of carbon atom (2) in hexonic acids has been definitely established by chemical methods. It thus became necessary to establish the genetic relationships between the hexonic and the amino-hexonic acids.

Table 10 represents the values and the direction of the rotation of carbon atom (2) in the hexonic and hexosaminic acids when the rotation of the hydrochlorides of the latter are compared with those of the phenyl-hydrazides of the former.

The similarity of the specific rotations is striking. However, this property alone is not sufficient to establish the configurational relationships of the two groups of substances. Therefore additional proof was needed. For this reason, the following points were investigated: first, the equilibrium between the two epimeric hexonic acids formed by the addition of prussic acid to a pentose; second. the equilibrium between the corresponding two hexosaminic acids formed through the addition of prussic acid to amino-pentoses. The two equilibria were then compared and found as given in table 11.

On the basis of the analogies recorded in tables 10 and 11, the relationships as given in table 12 could be formulated. Further it was found that when chitosaminic acid was deaminized, it gave gluconic acid (as the 2-5 anhydro form) but not mannonic. This was in agreement with Fischer's assumption that on deamination of α-amino acids with nitrous acid, a Walden Inversion occurs.

If, in addition, it is remembered that it was customary to designate as "d-amino acids" all such acids which rotate to the right in hydrochloric acid solution, and as "d-hydroxy acids" all acids of which the salts rotate to the right, it will become evident how the relationships expressed in table 12 for a time seemed logical and correct.

Doubts as to the rationality of this mode of comparison came to the fore when the directions of rotations of carbon atom (2) were determined not from the behavior of the sugar acids but from that of the sugars themselves. On the basis of van't Hoff's superposition theory it is possible, as was shown by Hudson, to determine the direction of the rotation of carbon atom (2) of two epimeric sugars

when the rotations of the α- β-forms of each are known.   When, on the one hand, two hexoses, glucose and mannose, were taken for comparison and on the other hand, two 2-amino-hexoses; chitosamine

TABLE 10.

ACID HYDROCHLORIDES	$[\alpha]_D$ OF CARBON ATOM (2)	$[M]_D^{20}$	PHENYL-HYDRAZIDE	$[\alpha]_D$ OF CARBON ATOM (2)	$[M]_D^{20}$
Epichitosaminic................	+12.5°	+24.37°	Gluconic	+14.25°	+42.18°
Chitosaminic..................	−12.5	−24.37	Mannonic	−14.25	−42.18
Dextro-xylohexosaminic.........	+12.5	+24.37	Gulonic	+14.25	+42.18
Levo-xylohexosaminic...........	−12.5	−24.37	Idonic	−14.25	−42.18
Epichondrosaminic.............	+12.5	+24.37	Galactonic	+8.25	+24.42
Chondrosaminic................	−12.5	−24.37	Talonic	−8.25	−24.42
Dextro-ribohexosaminic.........	+19.12	+37.28	Allonic	+20.8	+61.56
Levo-ribohexosaminic...........	−19.12	−37.38	Altronic	−20.8	−61.56

TABLE 11.

FROM	ROTATION OF CARBON ATOM (2) IN PRE-DOMINATING FORM	FROM	ROTATION OF CARBON ATOM (2) IN PRE-DOMINATING FORM
Arabinose....................	Levo	Aminoarabinoside...........	Levo
Lyxose......................	Levo	Aminolyxoside..............	Levo
Xylose......................	Dextro	Aminoxyloside..............	Dextro
Ribose......................	Levo	Aminoriboside..............	Levo

TABLE 12.

Chitosaminic acid...	2-aminomannonic acid
Epichitosaminic acid.....................................	2-aminogluconic acid
Chondrosaminic acid.....................................	2-aminotalonic acid
Epichondrosaminic acid...................................	2-aminogalactonic acid
Dextro-d-xylohexosaminic acid............................	2-aminogulonic acid
Levo-d-xylohexosaminic acid..............................	2-aminoidonic acid
Dextro-d-ribohexosaminic acid............................	2-aminoallonic acid
Levo-d-ribohexosaminic acid..............................	2-aminoaltronic acid

and epichitosamine (all may be taken in the form of pentacetates) then it was found that carbon atoms (2) in glucose and in chitosamine rotated in the same direction, namely, to the right, and man-

nose and epichitosamine both to the left. On the basis of these observations, chitosamine should be considered configuratively related to glucose, and epichitosamine to mannose. Thus, a contradiction arose between the conclusions reached on the basis of the optical rotations of the epimeric hexosaminic acids and those reached on the basis of the epimeric amino-hexoses. Further inquiry became necessary in order that a decision could be reached in favor of one or the other conclusion. It may be added here that the preponderance of evidence was found in favor of the second of the two named relationships.

It was known from the work of Hudson and his co-workers that the difference in the molecular rotations of the α- and β-glucoses had a normal value, and from the calculations of Hudson and Yanovski and from the experimental work of Levene it was definitely proved that in the case of the α- and β-mannoses, the corresponding value was abnormal. Some time ago, Irvine prepared the α- and β-isomers of chitosamine hydrochloride and found that the difference of the molecular rotation of the two forms was normal and therefore suggested that chitosamine was configurationally related to glucose. This evidence alone would scarcely be sufficient to establish the configuration of chitosamine. The molecular rotations of sugars are determined largely by the character of the oxidic ring and it is possible that in a simple sugar and in the corresponding 2-amino sugar the predominating form may have a different ring structure.

However, though insufficient in itself, the conclusion of Irvine found confirmation in the above-mentioned result of the evaluation of the rotation of carbon atom (2) of chitose on the basis of the rotations of α- and β-chitose pentacetates and α- and β-epichitose pentacetates. A third point of evidence favorable to this conclusion is found in the consideration of the properties of epichitosamine. The difference of the optical rotations of the α- and β-forms of this sugar was found abnormal, resembling mannose in this respect, and if the structure of 2-amino-mannose is attributed to epichitose, then the structure of 2-amino-glucose should be assigned to chitose. For this theory, then, three points of evidence have accumulated.

There still remained the puzzle of the discrepancy between the conclusions following from the observations made on the properties

TABLE 13.

Showing the Specific Rotations of the Free Acids and of Their Derivatives.
(All of the d series.)

	FREE ACIDS $[\alpha]_D^0$	Na SALTS $[\alpha]_D^{20}$	PHENYL-HYDRAZIDES $[\alpha]_D^{20}$	AMIDES $[\alpha]_D$
Gluconic................................	0.0°	+11.78°	+18.0°	+31.2°
Mannonic...............................	+15.6	−8.82	−10.5	−17.3
Idonic..................................	+	−2.52	−15.1	−
Gulonic.................................	−1.6	+12.68	+13.45	+15.2
Galactonic..............................	−8.0	+0.40	+12.2	+30.0
Talonic.................................	?	?	+4.35	−
Allonic.................................	−10.0	+4.30	+25.88	−
Altronic................................	+8.0	−4.05	−15.8	−

TABLE 14.

ACIDS	IN 5 PER CENT NaOH $[\alpha]_D^0$		IN 2.5 PER CENT HCl $[\alpha]_D^0$
Chitosaminic..............................	+1.3°	c = 5.0	−15.0°
Epichitosaminic...........................	−5.0	c = 5.0	+10.0
Dextro-d-xylo-2-aminohexonic...............	−16.0	c = 2.5	+14.0
Levo-d-xylo-2-aminohexonic.................	+2.0	c = 2.5	−11.0
Chondrosaminic...........................	−15.0	c = 2.5	−17.0
Epichondrosaminic.........................	+1.8 ,	c = 2.5	+8.0
Dextro-d-ribo-2-aminohexonic...............	+2.0·	c = 2.5	+12.5
Levo-d-ribo-2-aminohexonic.................	−15.0	c = 2.5	−26.0

TABLE 15.

First group	
Chitosaminic acid	Gluconic acid
Chondrosaminic acid	Galactonic acid
d-Levo-xylohexosaminic acid	Gulonic acid
d-Levo-ribohexosaminic acid	Allonic acid
Second group	
Epichitosaminic acid	Mannonic acid
Epichondrosaminic acid	Talonic acid
d-Dextro-xylohexosaminic acid`	Idonic acid
d-Dextro-ribohexosaminic acid	Altronic acid

of the sugars and those of the acids. However, a wholly harmonious theory is reached when a different basis is chosen for connecting the configurations of hexonic and amino-hexonic acids than the one taken for table 10.

A scrutiny of tables 13 and 14 showing the direction of the rotation of the hexonic acids in solution of dilute hydrochloric acid and the rotations of the metallic salts, phenyl-hydrazides or amides brings to light certain regularities. From these tables it is seen that all hexonic and all 2-amino-hexonic acids can be divided into two groups as given in table 15. In the first group, the metallic salts exhibit a higher dextro-rotation than the free acids; in the second, the order is reversed. Thus, accepting as a basis for classification the *sign* of the difference of the molecular rotations of the free acid and of its metallic salt, the contradiction is removed which existed between the conclusions reached on the basis of the properties of the amino-hexonic acids and those on the basis of the properties of amino-hexoses.

It was shown in the previous sections of this paper that the same rule is applicable for the classification into the d and l series of the simple amino and hydroxy acids as well as of the thio and sulfo acids. The observations on sugars and amino sugars, and on sugar acids and amino sugar acids, place this rule on a firmer basis.

The practical results of all the investigations in this field of endeavor, then, are the following:

1. All natural amino acids are configurationally related to l-hydroxy acids which, in their turn, are configurationally related to l-tartaric acid. The amino or hydroxyl groups in these acids have the same allocation as the hydroxyl on carbon atom (2) of mannose.

2. 2-Amino-hexoses occurring in nature, chitosamine and chondrosamine, have the configurations of 2-amino-glucose and of 2-amino-galactose respectively.

3. In α-amino acids, the deamination by means of nitrous acid takes place without Walden Inversion.

The evolution of these views was very gradual and many schools of thought contributed to it. Fischer's classical work and the work of Wohl and Freudenberg and Karrer have established by purely chemical methods the configurational relationships between members of the same classes of substances. Levene and Mikeska have studied

the influence on the optical rotation of the polarity of the groups attached to the asymmetric carbon atom. Frankland and others have studied the influence of structure on optical rotation. Clough, Karrer, Freudenberg and Levene have made use of these different methods and have added some new ones in order to correlate the configuration of α-amino acids and α-halogen acids with that of α-hydroxy acids and of nitrogenous sugars with simple sugars.

REFERENCES.

On Configurational Relationships of Hydroxy Acids.

BREMER, G. J. W.: *Ber. d. deut. chem. Ges.*, 8, 861 (1875).
FISCHER, E.: *Ber. d. deut. chem. Ges.*, 29, 1377 (1896). (Configuration of tartaric acid.)
FREUDENBERG, K.: *Ber. d. deut. chem. Ges.*, 47, 2027 (1914).
FREUDENBERG, K., AND BRAUNS, FRITZ: *Ber. d. deut. chem. Ges.*, 55, 1339 (1922).
WOHL, A., AND MOMBER, F.: *Ber. d. deut. chem. Ges.*, 50, 455 (1917).
WOHL, A., AND SCHELLENBERG: *Ber. d. deut. chem. Ges.*, 55, 1404 (1922).
WALDEN, P.: Optische Umkehrerscheinungen, Fried. Vieweg and Sohn Braunschweig (1919).

On Configurational Relationships of Amino Acids.

FISCHER, E., AND RASKE, K.: *Ber. d. deut. chem. Ges.*, 40, 3717 (1907); 41, 893 (1908).
KARRER, P., AND KAASE, W.: *Helv. Chim. Acta*, 6, 411 (1923); 6, 957 (1923).
WASER, E., AND BRAUCHLI, E.: *Helv. Chim. Acta*, 7, 740 (1924).

The Influence of External Conditions on Configurationally Related Substances.

ARMSTRONG, H. E., AND WALKER, E. E.: *Proc. R. Soc.*, A, 88, 388 (1913).
CLOUGH, G. W.: *Trans. Chem. Soc.*, 113, 526 (1918).
FRANKLAND, P., AND WHARTON, F. M.: *Trans. Chem. Soc.*, 69, 1587 (1896).
FREUDENBERG, K., AND RHINO, F.: *Ber. d. deut. chem. Ges.*, 57, 1547 (1924).
KARRER, P., AND KAASE, W.: *Helv. Chim. Acta*, 2, 436 (1919), 3, 248 (1920).
LOWRY, T. M.: *Trans. Chem. Soc.*, 103, 1067 (1913).
PATTERSON, T. S.: *Trans.* 77, 1106 (1900); 85, 1142 (1904); 92, 1843 (1908); 95, 323 (1909); 109, 1145 (1916); etc.
PICKARD, R. H., AND KENYON, J.: *Trans. Chem. Soc.*, 99, 45 (1911); 101, 620, 1427 (1912); 103, 1923 (1913); 105, 830 (1914); 105, 1115, 2226 (1914); etc.
RUPE, H., AND AKERMANN: *Ann. d. Chem.*, 420, 1 (1919).

Influence on the Optical Rotation of the Change of Polarity of One of the Groups Attached to the Asymmetric Carbon Atom.

LEVENE, P. A., AND MIKESKA, L. A.: *Jour. Biol. Chem.*, 59, 473 (1924); 60, 1, 685 (1924); 63, 85 (1924).

Configurational Relationships in 2-Amino-Hexoses and 2-Amino-Hexonic Acids.

LEVENE, P. A., AND CO-WORKERS: Monographs of The Rockefeller Institute for Medical Research, No. 18 (1922).
LEVENE, P. A.: *Jour. Biol. Chem.*, 59, 123 (1924); 63, 95 (1924).

Configurational Relationships of Simple Sugars and Sugar Acids Established on the Basis of van't Hoff's Superposition Theory.

FREUDENBERG, K., BRAUNS, F., AND SIEGEL, H.: *Ber. d. deut. chem. Ges.*, 56, 193 (1923).
HUDSON, C. S.: *J. Amer. Chem. Soc.*, 31, 66 (1909); 38, 1566 (1916); 39, 462 (1917); 40, 813 (1918).
HUDSON, C. S., AND YANOVSKI, E.: *Journ. Amer. Chem. Soc.*, 39, 1013 (1917).
HUDSON, C. S., AND KOMATSU, S.: *Journ. Amer. Chem. Soc.*, 41, 1141 (1919).
LEVENE, P. A.: *Jour. Biol. Chem.*, 23, 145 (1915).
LEVENE, P. A., AND MEYER, G. M.: *Jour. Biol. Chem.*, 26, 355 (1916); 31, 623 (1917).
WEERMAN, R. A.: Dissertation published by A. Kruyt, Amsterdam (1916).

THE ACTION OF ALKALIES ON PEPTIDES AND ON KETOPIPERAZINES.

BY P. A. LEVENE AND M. H. PFALTZ.

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In a previous communication[1] we reported on the action of weak and of stronger alkali on dextro-alanyl-dextro-alanine anhydride. It was observed that the anhydride was partially racemized if it was allowed to remain in an aqueous solution containing one equivalent of sodium hydroxide for 24 hours. When the concentration of the alkali was increased tenfold, the anhydride was rapidly converted into the dipeptide and the latter preserved its normal optical activity in the course of 72 hours. It was then suggested that, if found to be general, this peculiarity could have a theoretical and a practical value: first, it could be used for the detection of the presence of ketopiperazines in the protein molecule; secondly, it might furnish an explanation as to the mechanism of the racemization of proteins. Dakin was the first to advance a comprehensive theory of this phenomenon in protein. Kossel and Weiss prior to Dakin observed that when proteins are acted upon by half normal alkalies, certain of their component amino acids lose part or all of their optical activity, whereas other amino acids remain with their optical activity unimpaired. Dakin sought to explain this phenomenon by the assumption that only those amino acids are racemized which are situated between two other amino acids. Thus, in a tripeptide, only the central and not the terminal amino acids can be racemized. Dakin further postulated that the intermediate phase in the process of racemization consisted in an enolic tautomerization. Thus, considering the case of glycyl-levo-

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[1] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661.
All other references are contained in that publication.

alanyl-glycine, the entire process may be represented in the following way.

$$CH_2NH_2CO\cdot NHCHCO\cdot NHCH_2COOH \rightarrow CH_2NH_2CO\cdot NHC = C(OH)\cdot NHCH_2COOH$$

$$\underset{\text{levo}}{|}_{CH_3} \qquad\qquad\qquad\qquad |_{CH_3}$$

$$\rightarrow CH_2NH_2CONHCH(CH_3)CONHCH_2COOH$$

$$d, l \text{ form.}$$
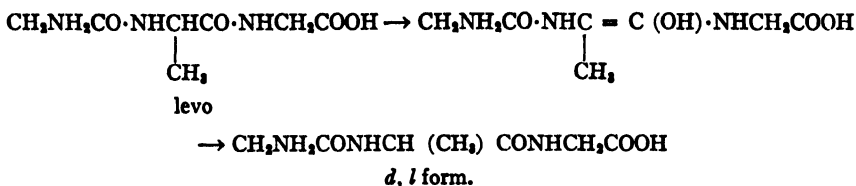
In our previous communication it' was shown that ketopiperazines represent a convenient system for enolization and that therefore when exposed to the action of alkali, they may suffer racemization as long as the ring structure remains intact. Thus it was suggested that in the case of alanyl-alanine anhydride the intermediate phase may have the following structure.

$$
\begin{array}{c}
C(OH) \\
\diagup\!\!\diagup \quad \diagdown \\
CH_3C \qquad NH \\
| \qquad\qquad | \\
NH \qquad CCH_3 \\
\diagdown \quad \diagup\!\!\diagup \\
COH
\end{array}
$$
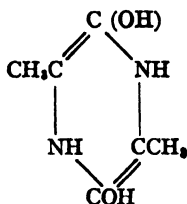
As another instance of racemization in a ketopiperazine may be quoted the prolyl-glycine anhydride isolated by Levene and Beatty from the products of tryptic digestion of gelatin. This ketopiperazine was nearly 75 per cent racemized.

To our knowledge, no experimental evidence exists as to the racemization of simple polypeptides. It is therefore expedient to test the influence of dilute alkalies on simple polypeptides and to compare it with that on ketopiperazines. Our views on the mechanism of racemization of protein and on the presence of ketopiperazines in the protein molecule will be greatly influenced by the outcome of such experiments.

In the present communication are reported the results of observations on one tripeptide, namely, glycyl-levo-alanyl-glycine; on one dipeptide, levo-alanyl-glycine, and on two ketopiperazines, namely levo-alanyl-glycine anhydride and levo-prolyl-glycine anhydride. The results of the observations are the following:

Tripeptide.—In dilute alkali (0.1 N) after 48 or 72 hours, there

was observed no hydrolysis of the peptide, no change in its optical rotation, and no change in the optical rotation of the alanine obtained from it. In a 3 per cent concentration in normal alkali in the same interval, there was observed a marked degree of hydrolysis and no trace of racemization of the alanine obtained on hydrolysis of the product of the action of the alkali.

Dipeptide.—In the course of 48 hours, a 1.5 per cent solution of the peptide in 0.1 N alkali remained unchanged with respect to rotation and hydrolysis. The alanine obtained on hydrolysis of the final product of the action of alkali had its original rotation of $[\alpha]_D^{25} = -14.3°$.

The same peptide in the same concentration in solution of N alkali, at the end of 48 hours suffered hydrolysis to the extent of 52 per cent, and the final product of the action of alkali on complete acid hydrolysis furnished alanine with the original rotation of $[\alpha]_D^{25} = -14.9°$. Thus, in the case of levo-alanyl-glycine, no racemization was observed under the conditions of the present experiment.

Ketopiperazines.—Alanyl-glycine anhydride. Three experiments were performed with this ketopiperazine. In one, the concentration of the peptide was 3 per cent in a solution containing 0.4 of an equivalent of alkali; in the second experiment, a 2 per cent solution of the ketopiperazine in a solution containing 0.7 of an equivalent was kept for 48 hours at 15°C. and in the third, the conditions were the same as in the second, except for the temperature, which was somewhat higher, namely, 18°C. In the first instance, the ketopiperazine was hydrolyzed into the dipeptide only to the extent of 30 per cent but no perceptible racemization was observed. The alanine obtained on complete acid hydrolysis had the specific rotation of $[\alpha]_D^{25} = -13.0°$.

In the second experiment at the end of 48 hours, all of the ketopiperazine was converted into the dipeptide and the rotation of the alanine obtained on hydrolysis was $-8.1°$. Finally, in the third experiment, only 72 per cent of the ketopiperazine was converted into the dipeptide and the alanine obtained from it had the specific rotation of $[\alpha]_D^{25} = -5.0°$.

In solutions containing four or eight equivalents of alkali, there was observed considerable hydrolysis also of the dipeptide. The alanine obtained from the final product had the optical rotation of $[\alpha]_D^{25} = -14.0°$.

Levo-Prolyl-Glycine Anhydride.—The mater ialused in these experiments was of the original ketopiperazine obtained by Levene and Beatty from the products of tryptic digestion of gelatin. It had a specific rotation of $[\alpha]_D^{20} = -66°$; hence, it was about 66 per cent racemized. The *l*-proline obtained from it had the specific rotation of -18.4 or was about 75 per cent racemized.

A 2 per cent solution of this ketopiperazine in a little less than one equivalent of alkali showed that after 24 hours 80 per cent of the anhydride was converted into the peptide. The rotation of the proline obtained on hydrolysis of the final material was $[\alpha]^{20} = -3.8°$.

The same substance in the same concentration but in a solution of normal alkali showed 96 per cent conversion into the dipeptide and the proline obtained from it had a specific rotation of $[\alpha]_D^{20} = -3.7°$ Thus this ketopiperazine seems to be more resistant towards the hydrolytic action of alkali and hence is racemized by strong as well as by weak alkali.

<div align="center">CONCLUSIONS.</div>

1. The tripeptide glycyl-levo-alanyl-glycine in solution of either one or ten equivalents of alkali does not undergo racemization on standing.

2. The dipeptide levo-alanyl-glycine under the conditions given in (1) does not undergo racemization.

3. In ketopiperazines, levo-alanyl-glycine anhydride and in levo-prolyl-glycine anhydride under the influence of dilute alkalies, racemization takes place.

4. Racemization in the present experiments was never complete. The degree of racemization seems to depend, on the one hand, on the stability of the ketopiperazine ring; on the other, on the concentration of the alkali.

5. The significance of these observations will depend on the outcome of the work on a larger number of polypeptides and ketopiperazines. The work is now in progress in this laboratory.

<div align="center">EXPERIMENTAL PART.</div>

All experiments were carried out in a uniform manner. The initial solution was made with the solvent cooled to about $+3°C$. The solu-

tion was then kept at a constant temperature of 18°C. In Experiment 9 the temperature was +15°C.

The rotations were measured for a short period at intervals varying from 15 to 60 minutes. These measurements, however, will not be recorded here in view of the fact that they are of little importance without simultaneous measurements of the ratios between the amino and the total nitrogen, which would have involved a great deal of work. At the end of each experiment such measurements were made and therefore both the optical rotations and nitrogen ratios are recorded.

At the end of each experiment, an aliquot part of the solution generally 75 per cent (15 cc. if the original solution was 20 cc.) was diluted with an equal volume of acid of the same normality as that of the alkali used in the experiment. These solutions were used for measurements of the rotations and the nitrogen ratios. From these data the extent of hydrolysis brought about by the alkali can be measured with a fair degree of approximation.

For hydrolysis an aliquot part of the neutral solution was concentrated and made up to a volume of 20 cc. of a solution containing 10 per cent of hydrochloric acid. This solution was heated in a sealed tube at 105°C. for 12 hours. It was found that at the end of that time, the hydrolysis was complete. The concentration of the important amino acid in this solution was calculated from these values.

All rotations were measured at D light in 200.0 mm. tubes. The experimental data are given in the table on the following page.

Name of substance.	(1) Concentration of substance. *per cent*	(2) Concentration of alkali.	(3) Volume. *cc.*	(4) α Of original solution.	(5) α At the end of experiment.	(6) After neutralization.	(7) $\frac{NH_2N}{T \cdot N}$ End of experiment. *per cent*	(8) α After hydrolysis.	(9) T·N in portion hydrolyzed in 20 cc. *gm.*	(10) $[\alpha]_D^{25}$ active amino acid.
Glycyl-levo-alanyl-glycine.	(1) 3	N/1	20	+2.92°	+1.60°	+2.00°	63.0*	−0.25	0.0742	−14.5°†
"	(2) 3	N/1	20	+3.13	+3.15	+3.80°	42.0	−0.23	0.0742	−13.9
Levo-alanyl-glycine.	(3) 1.5	N/1	20	−0.25	−0.20	−1.08	73.0	−0.17	0.0546	−14.9
"	(4) 1.5	N/10	20	−0.33	−0.30	−1.42	48.5	−0.16	0.0525	−14.3
Levo-alanyl-glycine anhydride.	(5) 3	N/1	20	+0.80	+0.82	+1.00	65.3	−0.35	0.0826	−13.3
"	(6) 3	N/20	20	+0.95, +0.02	+0.35	+0.36	16.9	−0.35	0.0854	−12.8
"	(7) 1.5	N/1	20	+0.20, +0.50	+0.43	+0.50	78.0	−0.17	0.0406	−14.0
"	(8) 2	N/10	30	—	+0.40	+0.32	48.5	−0.25	0.0938	− 8.1
"	(9) 2	N/10	20	−0.14	+0.45	+0.52	37.6	−0.10	0.0588	− 5.0
Levo-prolyl-glycine anhydride.	(10) 2	N/1	20	−0.50	−0.11	−0.32	48.5	−0.08	0.0490	− 3.7‡
"	(11) 2	N/10	20	−1.28, −0.82	−0.09	−0.22	40.0	−0.08	0.0476	− 3.8

*The rotation of the alanine obtained on direct hydrolysis of the peptide without preliminary treatment with alkali = $[\alpha]_D^{25} = -14.5°$.

†The specific rotation of the ketopiperazine was $[\alpha]_D^{25} = -66.0°$ (in water). The proline obtained from it on direct hydrolysis had a specific rotation of $[\alpha]_D^{25} = -18.4°$.

‡All amino-nitrogen estimations were made in Van Slyke's apparatus.

SYNTHETIC NUCLEOSIDES.

I. THEOPHYLLINE PENTOSIDES.

BY P. A. LEVENE AND HARRY SOBOTKA.

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Our knowledge of the details of the structure of purine nucleosides in based principally on analytical data. A recent publication of P. A. Levene (1) in which the methylation of xanthosine by means of diazomethane was reported gave additional data. All the analytical figures seem to indicate that in purine nucleosides the sugar is attached to atom (7) of the purine.

Evidence in favor of this view may be found in the synthetic work of E. Fischer and B. Helferich (2, 3, 4). These workers, immediately after the discovery of the nucleosides by Levene and Jacobs, undertook the synthesis of these substances. A review of the entire series of substances prepared by Fischer and his coworkers brings out the fact that they may be classified in two groups. Substances of one group resemble natural nucleosides, whereas others are distinctly different.
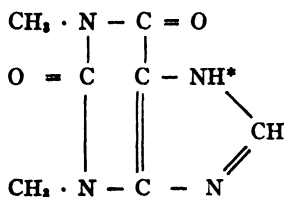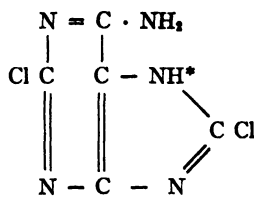
In the case of 1,3-dimethyl-2,6-dioxy purine (theophylline) (Formula I) and of 2,8-dichloroadenine (II) only the hydrogen in position (7), marked by an asterisk(*), can be replaced; position (9), though possible, is less probable. Hence, there can be no doubt

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as to the place of substitution.   On the other hand, in theobromine (III) and in hydroxycaffeine (IV):

$$
\begin{array}{ll}
\text{HN} - \text{C} = \text{O} & \text{CH}_2 \cdot \text{N} - \text{C} = \text{O} \\
\quad | \qquad | & \qquad | \qquad | \\
\text{O} = \text{C} \quad \text{C} - \text{N} \cdot \text{CH}_3 & \text{O} = \text{C} \quad \text{C} - \text{N} \cdot \text{CH}_3 \\
\quad | \qquad \| \qquad \searrow & \qquad | \qquad \| \qquad \searrow \\
\quad | \qquad \| \qquad \text{CH} & \qquad | \qquad \| \qquad \text{C} = \text{O} \\
\quad | \qquad \| \qquad \diagup\!\diagup & \qquad | \qquad \| \qquad \diagup \\
\text{CH}_3 \cdot \text{N} - \text{C} - \text{N} & \text{CH}_3 \cdot \text{N} - \text{C} - \text{NH} \\
\qquad \text{III.} & \qquad \text{IV.}
\end{array}
$$
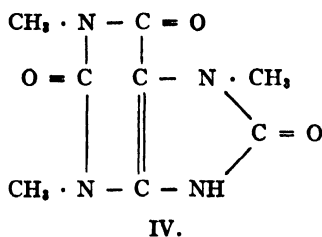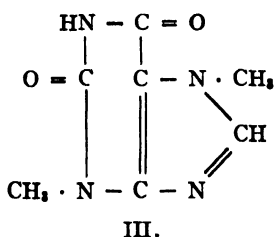
the free NH group (positions (1) and (9) respectively) is linked to one carbonyl in position (8) (in IV) or to two carbonyls in positions (2) and (6) (in III).   Thus, during the formation of the silver salt enolization may take place, in which case the condensation leads to substitution in one of the C-O- groups.

The glucosides of theobromine and hydroxycaffeine belong to the second class of substances.   They are extremely unstable even towards boiling water.   The tetracetylglucoside of hydroxycaffeine was found to be so unstable that it could not be deacetylated at all without opening the sugar purine linkage.   Thus these substances behaved unlike the natural nucleosides.

The synthetic glucosides of theophylline and dichloroadenine belong to the other class.   The latter was used for the preparation of adenine, hypoxanthine, and guanine glucosides.   The substances of this group showed a marked resistance both towards acid hydrolysis and towards alkaline hydrolysis.   In this respect these substances resembled the naturally occurring nucleosides.   However, this work of Fischer and his coworkers dealt principally with hexose nucleosides and only in a minor degree with pentose nucleosides.   And yet, for the present, more importance is attached to the latter, inasmuch as for the present nucleosides have been isolated only from pentose nucleic acid.   It seemed particularly expedient to prepare the pentosides of theophylline, in view of the fact that, as already mentioned, the natural guanosine had been converted into dimethyl xanthosine.

The xyloside and riboside of theophylline have now been prepared synthetically by us and the properties of the latter have been com-

pared with those of the dimethyl xanthosine. The identity of both substances was proved by the uniformity of their optical rotation and by the agreement of the rate with which they underwent acid hydrolysis.

EXPERIMENTAL PART.

Acetobromoxylose (5).—Xylose tetracetate was dissolved in two to three times its weight of a commercial solution of hydrobromic acid in glacial acetic acid. After standing $1\frac{1}{2}$ hours at room temperature 3 parts of chloroform were added, and the mixture was washed with ice water until neutral to Congo red. The chloroform solution was dried over $CaCl_2$ sticks and was then concentrated under reduced pressure. The viscous residue was taken up in a small amount of ether. After standing a few hours at 0°C., large transparent needles of the acetobromoxylose were collected. The yield was 65 per cent of the starting material or 60 per cent of the theory.

Acetobromoribose.—This substance had not as yet been prepared. It has less favorable properties than the analogous xylose derivative. When working with small quantities, it is advantageous to use 5 parts of HBr solution and 1 part of tetracetate. The substance should be washed not more than twice, then the dried chloroform solution is concentrated under reduced pressure, care being taken that the distillation is interrupted as soon as a slight darkening begins. An excess of absolute petrolic ether (b.p. 40–60°) is added to the concentrated solution. Due to its hygroscopic character, the product, which crystallizes in the cold, cannot be filtered on a Büchner funnel but must be centrifugalized and the centrifuge bottles placed at once in a vacuum desiccator over phosphorus pentoxide, soda-lime, and solid paraffin. Yield 50 to 55 per cent of the theory.

0.1814 gm. substance required 5.30 cc. 0.1 N $AgNO_3$: 0.04235 gm. Br.
$C_{11}H_{15}O_7$ Br (339.04).   Calculated.   Br 23.57.
Found.   "   23.35.

Triacetyl Theophylline Xyloside.—According to the directions given by E. Fischer and B. Helferich, 17 gm. of powdered theophylline silver were suspended in a solution of the calculated amount (19 gm.) of acetobromo-*d*-xylose in 150 cc. of xylene and boiled under a reflux condenser until a sample of the liquid gave no more reaction for

bromine. The solution was filtered into another 150 cc. of cold xylene. By adding an excess of petrolic ether a white flocculent precipitate of triacetyl theophylline xyloside settled out. The compound was found to be very soluble in water, alcohol, ether, and toluene, but less soluble in ethyl acetate. The yield 16 gm. = 65 per cent of the theory. The rotation in methyl alcohol was

$$[\alpha]_D^{25} = \frac{-0.93° \times 100}{1 \times 4.25} = -21.9°$$

0.0983 gm. substance: 0.1766 gm. CO_2 and 0.0470 gm. H_2O.
$C_{18}H_{22}O_9N_4$ (438.21). Calculated. C 49.29, H 5.07.
Found. " 48.99, " 5.35.

TABLE I.

Hydrolysis of a 2.45 Per Cent Solution of Theophylline Xyloside in 0.1 N HCl at 100°C.

Time.	α_D ($l = 2$dm.)	Hydrolysis.	$k \times 10^4$
min.		*per cent*	
0	−1.42	0	
200	−0.98	23.5	5.82
350	−0.66	40.6	6.47
500	−0.44	52.4*	6.44
∞	+0.45	100	

* 0.1 cc. of the solution at this point gave with the Shaffer-Hartmann method (6) 0.75 mg., calculated for glucose corresponding to 0.625 mg. of pentose. At 100 per cent hydrolysis 1.178 per cent of xylose would be released so this value of 53 per cent of sugar is in good accord with the optical determination.

Theophylline Xyloside.—10 gm. of the acetyl compound were dissolved in absolute methyl alcohol and a current of ammonia was passed through the thoroughly cooled solution. Overnight a hard white crust was formed, which gave after repeated recrystallization from methyl alcohol and water more than 4 gm. (60 per cent of the theoretical amount) of felt-like needles which melted at 229° (corrected). The substance analyzed as follows:

0.1013 gm. substance: 0.1704 gm. CO_2 and 0.0472 gm. H_2O.
0.0934 " " required (Kjeldahl) 12.10 cc. 0.1 N acid.
$C_{12}H_{16}O_6N_4$ (312.16). Calculated. C 46.14, H 5.16, N 17.95.
Found. " 45.88, " 5.21, " 18.13.

The optical rotation which was

$$[\alpha]^{25} = \frac{-0.57° \times 100}{2 \times 1.00} = -28.5° \text{ (in methyl alcohol)}$$

$$[\alpha]^{25} = \frac{-0.99° \times 100}{1 \times 3.61} = -27.4° \text{ (in water)}$$

remained unchanged in 0.5 N acid solution. In 0.5 N alkali the rotation became

$$[\alpha]_D^{25} = \frac{-1.48° \times 100}{2 \times 1.805} = -41.0°$$

The rate of hydrolysis is seen from Table I.

Triacetyl Theophylline Riboside.—5 gm. of freshly prepared aceto-bromoribose and 5 gm. of theophylline silver in 50 cc. of xylene were treated as above. The yield was 3.5 gm. The analysis showed that considerable amounts of the solvent were retained.

$$[\alpha]_D^{25} = \frac{-0.66° \times 100}{2 \times 7.76} = -4.25° \text{ (in methyl alcohol)}$$

Theophylline Riboside.—The crude triacetate was deacetylated by means of NH_3 in methyl alcohol. Like the other ribose derivatives, the theophylline riboside differs from the corresponding derivatives of other pentoses, particularly from those of xylose, by its extremely high solubility and hygroscopic character. Since also with prolonged standing no precipitate was formed in the methyl alcoholic solution, both the ammonia and the alcohol were driven off under reduced pressure. The viscous residue when redissolved in ethyl alcohol and precipitated with ether formed an amorphous flocculent deposit which retained about 1 molecule of ethyl alcohol. After standing several weeks in the refrigerator, spherical aggregates of hard yellow crystals were obtained from the mother liquor. They melted at 234°C. (corrected) and gave the following analytical values.

0.1060 gm. substance: 0.1812 gm. CO_2 and 0.0524 gm. H_2O.
0.0948 "   "   : 0.1604 "   "   " 0.0482 "   "
0.0980 "   "   required (Kjeldahl) 12.25 cc. 0.1 N acid.
$C_{12}H_{16}O_6N_4$ (312.16).   Calculated.   C 46.14, H 5.16, N 17.95.
                          Found.   " 46.61, " 5.53, " 17.50.
                          "   " 46.14, " 5.58.

The rotation was

$$[\alpha]_D^{25} = \frac{-0.39° \times 100}{2 \times 0.925} = -21° \text{ (in ethyl alcohol)}$$

$$[\alpha]_D^{25} = \frac{-0.38° \times 100}{2 \times 0.495} = -38° \text{ (in 0.5 N NaOH)}$$

The rotation of a sample of dimethyl xanthosine from natural xanthosine was newly found to be

$$[\alpha]_D^{25} = \frac{-0.58° \times 100}{2 \times 1.245} = -23° \text{ (in ethyl alcohol)}$$

Comparison of the Rate of Hydrolysis.—Comparison of the rate of hydrolysis in the case of the theophylline riboside and in that of dimethyl xanthosine could not be carried out polarimetrically due to the small change in rotation. Because of the good checks obtained in the case of the xyloside by the use of the Shaffer titrimetric method (6), we followed the reaction by this method.

TABLE II.

Hydrolytic Action of 0.1 N HCl at 100°C.

Time.	Volume applied.	Pentose.	Hydrolysis.	$k \times 10^4$
On 1.85 per cent solution of theophylline riboside.				
min.	*cc.*	*mg.*	*per cent*	
120	0.2	0.335	18.7	7.50
On 2.595 per cent solution of theophylline riboside.				
60	0.4	0.55	11.0	7.81
120	0.4	0.88	17.6	7.00
240	0.2	0.79	31.6	6.87
On 2.565 per cent solution of dimethyl xanthosine.				
120	0.4	0.80	16.3	6.44
240	0.2	0.85	34.6	7.68

BIBLIOGRAPHY.

1. Levene, P. A., *J. Biol. Chem.*, 1923, lv, 437.
2. Fischer, E., and Helferich, B., *Ber. chem. Ges.*, 1914, xlvii, 210.
3. Fischer, E., and von Fodor, K., *Ber. chem. Ges.*, 1914, xlvii, 1058.
4. Helferich, B., and von Kühlewein, M., *Ber. chem. Ges.*, 1920, liii, 17.
5. Dale, J. K., *J. Am. Chem. Soc.*, 1915, xxxvii, 2745.
6. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 365.

SYNTHETIC NUCLEOSIDES.

II. SUBSTITUTED URACIL XYLOSIDES.

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In the preceding paper the identity of a synthetic purine nucleoside with a derivative of a genuine nucleic acid was discussed. In the present paper we give details of a series of experiments carried out with derivatives of uracil.

Although Fischer successfully synthesized purine glucosides, he was less fortunate in his attempts at preparing the corresponding pyrimidine derivatives (1, 2). Neither from 4-methyluracil nor from cytosine, thiouracil, or ethyl thiouracil could substances be synthesized similar to the natural uridine and cytidine. Contrary to their prototype, all of the synthetic products reduced Fehling's solution. Their extreme instability makes it evident that the carbohydrate component is not linked with either of the nitrogen atoms.

On comparing the formulæ for purine derivatives and for uracil, it is evident that in the ring of the latter derivative the chances for coupling with a non-enolizing imide group are not the same as in the case of theophylline.

$$
\begin{array}{llll}
1. & HN - C = O & 6. \\
   & \quad | \quad\quad | \\
2. & O = C \quad CH & 5. \\
   & \quad | \quad\quad || \\
3. & HN - CH & 4.
\end{array}
$$

I.
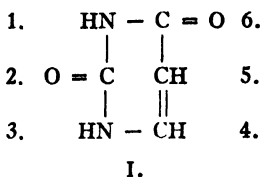
Through the alternation of CO and NH groups in the uracil ring there is always a two-fold possibility for substitution, as is the case with theobromine and hydroxycaffeine. The difficulties in con-

trolling the course of substitution even in partially substituted uracils are seen from the following figures.

$$
\begin{array}{ccc}
\text{CH}_3 \cdot \text{N} - \text{C} = \text{O} & \text{HN} - \text{C} = \text{O} & \text{N} - \text{C} \cdot \text{NH}_2 \\
\mid \quad \mid & \mid \quad \mid & \mid \quad \mid \\
\text{O} = \text{C} \quad \text{CH} & \text{C}_2\text{H}_5 \cdot \text{SC} \quad \text{CH} & \text{O} = \text{C} \quad \text{CH} \\
\mid \quad \parallel & \parallel \quad \parallel & \mid \quad \parallel \\
\text{HN} - \text{CH} & \text{N} - \text{CH} & \text{HN} - \text{CH} \\
\text{II.} & \text{III.} & \text{IV.}
\end{array}
$$

$$
\begin{array}{c}
\text{RN} = \text{C} = \text{O} \quad (\text{R} = \text{H or CH}_3) \\
\mid \quad \mid \\
\text{O} = \text{C} \quad \text{C} \cdot \text{NO}_2 \\
\mid \quad \parallel \\
\text{HN} - \text{CH} \\
\text{V.}
\end{array}
$$
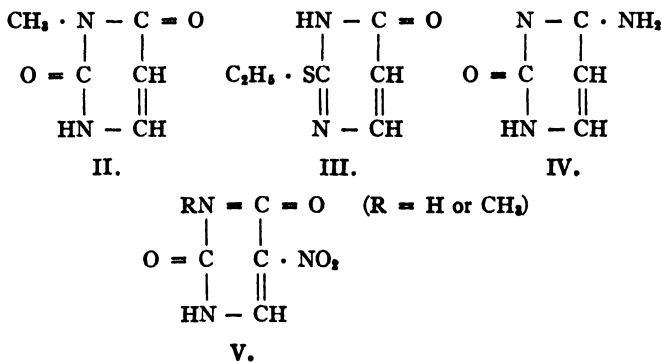
Substitution on carbon atoms (2) or (6) by monovalent groups such as alkoxyl, mercapto (III), or amino (IV) groups prevents further reaction of the adjacent nitrogen; a replacement of the oxygen by the bivalent sulfur tends to promote enolization, whereas substitution of two monovalent groups in the carbonyl group cannot be accomplished. In our attempt, we, nevertheless, varied both the substituting groups and their positions over a large range to cover any possible change in reactivity of the molecule.

Both *1-methyluracil and cytosine* (II and IV) were chosen for their free CO-NH group in position (2, 3).

The *5-nitrouracil* V which can be substituted in position (1), (2), (3), or (4) shows a remarkably strong acidic character which might possibly affect the relative reactivity of those positions.

In the *2-ethyl thio 6-oxy pyrimidine* (III) the free CO-NH group is placed in position (1, 6). The possibility is not excluded that an isomer exists having a double bond between atoms (1) and (2), which would permit substitution in position (3).

Finally, in the acidic *1-methyl-5-nitrouracil* (V; R = CH$_3$) replaceable hydrogen atoms are left in position (2) or (3).

Despite the varying character of the substituents, the reaction of acetobromopentose upon the silver compounds led to the formation of only 2- and 6-linkages; this was made evident by the readiness with which they underwent both acid and alkaline hydrolysis, the latter suggesting a close relationship to the class of imido esters. It is remarkable that cytosine did not react at all.

Having failed to obtain substances possessing the same stability as genuine uridine we attempted to employ for synthesis the sodium and potassium salts in place of the silver salts of pyrimidines. T. B. Johnson and F. W. Heyl (3) have shown that whereas the silver compound of 2-anilidouracil reacts with methyl iodide to give only 6-methoxy-2-anilidouracil, the potassium salt gave a mixture of 6-methoxy and 1-methyl-6-hydroxy compounds. Although well defined potassium salts were prepared from the acidic 5-nitro- and 1-methyl-5-nitrouracil, only the undesired isomeric pentosides were formed.

From the above figures it is seen that the sugar group is attached in the different pentosides in the following way. In the 1-methyl-5-nitrouracil, in position (2); in the 2-ethyl thio compound, probably in position (6); in the 5-nitrouracil, the sugar group may enter in either position (2) or (6). The same possibility also exists in 1-methyl uracil. In the two latter instances, no crystalline products could be obtained, even when the potassium salt of nitrouracil was prepared from nitrouracil-4-carboxylic acid. This fact seems to indicate that mixtures of the two isomers were formed.

Rate of Hydrolysis of the Nucleosides.—The course of acid hydrolysis has been followed by the polariscopic method in view of the fact that boiling with Fehling's solution causes by itself complete hydrolysis of these nucleosides. All xylosides under investigation exhibit a dextrorotation, except the nitro compounds; in this point at least, one is reminded of the natural pyrimidine nucleosides. The mercapto compound resembles natural uridine (4) in that its dextrorotation is increased in acid solutions.

It may be mentioned that no rise in dextrorotation during the earlier stages of hydrolysis has been observed which fact indicates the absence of the α-xylosides. This corroborates the general rule that acetobromo sugars always furnish β-glucosides.

On comparing the rates of hydrolysis, it was found that the 1-methyluracil xyloside was relatively stable.[1] No change in rotation took place in 0.2 N HCl within 3 hours at the temperature of

[1] The methyl compound shows a slightly higher stability also on boiling in alkaline copper solution than do the other derivatives.

the boiling water bath.   N acid under these conditions brings about 90 per cent hydrolysis.

Nitrouracil, though unaltered by 0.2 N acid in the cold, undergoes approximately 4 per cent hydrolysis within 2 hours at 100°. Its acetyl compound is even less stable, for it is hydrolyzed 70 per cent after 1 hour under the same conditions.

The mercapto compound shows still greater instability.   After the initial immediate increase in rotation with 0.2 N hydrochloric acid in the cold, a slow drop in rotation is to be observed which becomes constant after 2 hours but on heating the solution a further drop takes place and hydrolysis is complete after another 2 hours.   We do not attribute this effect to the existence of two compounds of different stability for even the second stage of hydrolysis is far too rapid to indicate a nitrogen linkage. The strong odor of mercaptan already developed with cold acid gives a clew to the first drop in rotation, which might be explained by the elimination of the mercapto group.

From these observations the possibility suggests itself that linkage in position (6) is less stable than the one in position (2).

The essential differences in the stability of the nucleosides synthesized by us, and of the natural nucleosides, and furthermore, the successful efforts of synthesis with theophylline, which does not permit of an oxygen linkage, corroborates the uridine formula which has been given by one of us in 1913 (5) and which recently has been confirmed from quite another point of view (6).

EXPERIMENTAL PART.

Reaction between Acetobromoxylose and Silver Compounds of Uracil Derivatives.[2]—Silver compounds from uracil and its derivatives are obtained by mixing their hot dilute aqueous solutions with the calculated amount of dilute silver nitrate solution.   The liberated nitric acid is carefully neutralized by ammonia.   The white precipitates

---

[2] The uracil derivatives have been prepared according to the directions given by H. L. Wheeler and H. F. Merriam (7) (2-mercaptouracil), T. B. Johnson and F. W. Heyl (3) (1-methyluracil), H. Biltz and M. Heyn (8) (5-nitrouracil), and R. Behrend and M. Lehmann (9) (1-methyl-5-nitrouracil).

are subsequently washed with water, alcohol, and ether and should be protected from the sunlight. Theoretical yields are obtained.

Ethyl Thiouracil Silver. 0.1008 gm. substance: 0.0413 gm. Ag.

$C_6H_7ON_2SAg$ (263.02). Calculated. Ag. 41.02.

Found. " 40.96.

1-Methyluracil Silver. 0.1015 gm. substance: 0.0466 gm. Ag.

$C_5H_5O_2N_2Ag$ (232.94). Calculated. Ag. 46.31.

Found. " 45.91.

Cytosine Silver. 0.1002 gm. substance: 0.0493 gm. Ag.

$C_4H_4ON_3Ag$ (217.94). Calculated. Ag. 49.50.

Found. " 49.20.

These compounds were added in a fine powdered state to a solution of acetobromoxylose in xylene. Boiling under reflux condenser is continued until the liquid gives no more halogen reaction. The residue is separated and twice more extracted by means of hot xylene.

2-Ethyl Thiouracil Triacetyl Xylose.—The compound was thus prepared from 13 gm. of the silver salt of 2-ethyl thiouracil and 17 gm. of acetobromopentose. The xylene was removed from the filtrate under diminished pressure. By alternating recrystallization from ethyl and methyl alcohol 11 gm. (55 per cent of the theory) of the substance melting at 104–105°C. (all melting points corrected) were obtained.

0.1000 gm. substance: 0.1790 gm. CO_2 and 0.0478 gm. H_2O.

0.1094 " " : 6.20 cc. nitrogen gas at 24°C. and 768.9 mm.

$C_{17}H_{22}O_8N_2S$ (414.26). Calculated. C 49.24, H 5.35, N 6.76.

Found. " 48.81, " 5.03, " 6.59.

$$[\alpha]_D^{25} = \frac{+1.36° \times 100}{1 \times 4.795} = +28.4°$$

Partial hydrolysis of this compound by methyl alcoholic ammonia led to the formation of *ethyl thiouracil xyloside* which, after removal of the ammonia and most of the alcohol, crystallized. Recrystallization from ethyl alcohol yielded white needles, fairly soluble in water. Melting point 114–115°C.

0.0958 gm. substance: 0.1594 gm. CO_2 and 0.0482 gm. H_2O.

0.0932 " " : 7.60 cc. nitrogen gas at 24°C. and 762.8 mm.

$C_{11}H_{16}O_5N_2S$ (288.15). Calculated. C 45.81, H 5.60, N 9.72.

Found. " 45.37, " 5.63, " 9.40.

The rotation in methyl alcohol was

$$[\alpha]_D^{25} = \frac{+1.20° \times 100}{1 \times 5.58} = +21.5°$$

The substance is very unstable towards alkali. 2 minutes boiling with Fehling's solution released 55 per cent of the reducing sugar.

By addition of 0.1 cc. of concentrated HCl to 3 cc. of the solution in methyl alcohol, the rotation increased immediately to

$$[\alpha]_D^{25} = \frac{+1.87° \times 100}{1 \times 5.40} = +34.6°$$

A solution in 0.2 N HCl decreased from the initial rotation of 0.73° during a few hours to 0.51°. At this point it stopped, but by heating to 100°C. for 2 hours, the value calculated for equilibrium xylose was attained, the pyrimidine base itself having no optical activity.

$$[\alpha]_D^{25} = \frac{+0.73° \times 100}{1 \times 2.30} = +31.7°$$

Final value found 0.18°; the calculated rotation for a 1.20 per cent solution of xylose would be

$$\alpha_D = \frac{+19° \times 1.20}{1 \times 100} = +0.23°$$

9 gm. of acetobromoxylose and 6 gm. of 1-methyluracil silver yielded 4 gm. of *1-methyluracil xylose triacetate* which settled down from the xylene solution. (Neither from the mother liquor nor from further extracts of the silver bromide residue could more of the product be obtained. Here as well as in the following experiments only amorphous masses could be extracted, the high nitrogen content of which despite repeated reprecipitations indicated that they consisted chiefly of uncombined base.)

0.1019 gm. substance: 0.1874 gm. CO_2 and 0.0486 gm. H_2O.
$C_{16}H_{20}O_9N_2$ (384.18).   Calculated.   C 49.98, H 5.25.
Found.   " 50.14, " 5.33.

In 2 per cent solution in the 2 dm. tube no optical activity was detected. By removal of the acetyl groups, the *1-methyluracil*

xyloside was formed which reduced Fehling's solution like the mercapto compound.

0.0993 gm. substance: 0.1662 gm. CO_2 and 0.0512 gm. H_2O.
0.1000 "   "   required (Kjeldahl) 8.55 cc. 0.1 N acid.
$C_{10}H_{14}O_6N_2$ (258.13).   Calculated.   C 46.49, H 5.47, N 11.37.
                                  Found.   " 45.63, " 5.76, " 11.97.

This substance had the following rotation in methyl alcohol.

$$[\alpha]_D^{25} = \frac{+ \, 0.56° \times 100}{1 \times 2.05} = + \, 27.3°$$

Neither at normal temperature nor at 100°C. were the substance and its optical activity affected by 0.2 N HCl within 3 hours. At least N acid acting on the xyloside in 1.17 per cent concentration for 3 hours at 100°C. caused a drop in rotation from 0.64° to 0.30° (2 dm.), the final value for 100 per cent liberated xylose figuring at 0.26°.

5-Nitrouracil Acetyl Xyloside.—5-Nitrouracil silver reacted with the acetobromo sugar to form an ochre-colored product, which was but slightly soluble in xylene. The yield was increased by alcoholic extraction of the solid residue. The substance could not be purified by recrystallization for its solutions in methyl alcohol underwent spontaneous decomposition above 60°C., apparently due to a reaction between NO_2 group and sugar radicle.

The same difficulty arose on deacetylation, unless the reaction was carried out with small quantities. The *5-nitrouracil xyloside* despite repeated recrystallization from ethyl alcohol and ethyl acetate could not be obtained in an analytically pure state. It reduces, however, larger amounts of alkaline copper solution than could be explained by assuming a contamination with uncombined xylose.

$$[\alpha]_D^{25} = \frac{- \, 0.22° \times 100}{2 \times 6.12} = - \, 1.80°$$

After 2 hours boiling with 0.2 N HCl the rotation increased to −0.18°; after 4 hours, to −0.15°, corresponding to 4 and 7 per cent hydrolysis respectively. (Final value +0.82°.)

Cytosine silver, with acetobromoxylose, did not form a condensation product containing nitrogen. Despite the precautions against

moisture taken throughout these experiments in the case of cytosine considerable quantities of *triacetyl xylose* crystallized from the xylene, forming spherical aggregates almost invisible in the solvent by their similar index of refraction. There was no sharp melting point as the material was an α, β equilibrium mixture.

0.1063 gm. substance: 0.1820 gm. CO_2 and 0.0554 gm. H_2O.

$C_{11}H_{16}O_8$ (276.13).   Calculated.   C 47.80, H 5.84.

Found.   " 47.90, " 5.99.

$$[\alpha]_D^{25} = \frac{+ 5.24° \times 100}{2 \times 4.976} = + 52.65° \text{ (water; final value)}$$

In other instances, however, where no synthesis was obtained, considerable quantities of acetobromo sugar were recovered in crystalline state, thus showing the absolute absence of water.

Alkali Compounds of Uracils.—These were obtained by dissolving the uracil derivatives in aqueous or alcoholic alkali hydroxide. They are extremely soluble and by evaporation form a syrup and finally hard crusts. Although a slight excess of alkali was used, the values for Na or K were always found to be too low. Even the mercaptouracil potassium which crystallized from concentrated solution, in long silk-like needles, after drying under vacuum at the temperature of boiling xylene retained considerable amounts of water, which was fatal for the condensation with acetobromo sugar.

Both the sodium and potassium salts of ethyl thiouracil in xylene as well as in absolute ethyl alcohol failed tó react with the sugar but left residues of the unchanged base. Besides, white platelets were precipitated in one instance from ethyl acetate with petroleum ether, which analyzed for an addition product $C_6H_8O_2N_2S \cdot HBr$.

No satisfactory results could be obtained with the potassium salt of 5-nitrouracil, prepared from the 5-nitrouracil-4-carbonic acid by heating its potassium salt to 170°C. The amorphous product had a specific rotation of about $-50°$. The optical activity was lost by heating 3 hours with 0.2 N acid to 100°C., due to hydrolysis and possibly decomposition of the sugar by freed nitric acid.

The beautiful slightly green potassium salt of 1-methylnitrouracil, when brought in contact with the acetobromoxylose yielded

greenish crystals, which melted at 243°C. (corrected) and analyzed for methyl nitrouracil triacetyl xyloside.

0.0980 gm. substance: 0.0424 gm. K_2SO_4.

$C_5H_4O_4N_2K$ (209.16).   Calculated.   K 18.69.
                              Found.   " 18.25.

0.1006 gm. substance: 0.1640 gm. CO_2 and 0.0416 gm. H_2O.
0.1000 "   "   : 8.90 cc. nitrogen gas at 25°C. and 764.5 mm.

$C_{16}H_{19}O_{11}N_3$ (429.18).   Calculated.   C 44.74, H 4.46, N 9.79.
                                     Found.   " 44.45, " 4.62, " 10.24.

This substance also reduces Fehling's solution. It is almost insoluble in water and alcohol and had the following rotation in pyridine methyl alcohol 1:1.

$$[\alpha]_D^{25} = \frac{-0.76° \times 100}{2 \times 0.836} = -45.5°$$

BIBLIOGRAPHY.

1. Fischer, E., and Helferich, B., *Ber. chem. Ges.*, 1914, xlvii, 210.
2. Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 1377.
3. Johnson, T. B., and Heyl, F. W., *Am. Chem. J.*, 1907, ᵛxxxvii, 637.
4. Levene, P. A., *J. Biol. Chem.*, 1920, xli, 492.
5. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1912–13, xiii, 507.
6. Levene, P. A., *J. Biol. Chem.*, 1925, lxiii, 653.
7. Wheeler, H. L., and Merriam, H. F., *Am. Chem. J.*, 1903, xxix, 484.
8. Biltz, H., and Heyn, M., *Ann. Chem.*, 1917, ccccxiii, 110.
9. Behrend, R., and Lehmann, M., *Ann. Chem.*, 1889, ccliii, 65, 77.

THE CONCENTRATION OF VITAMIN B. II.

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In a previous publication[1] it was shown that active growth-promoting material contained in brewers' yeast consisted of a mixture of organic and mineral substances even after it was concentrated to such a degree that quantities of it containing 0.06 mg. of nitrogen per day were sufficient to promote growth. It was then realized that further progress of the work would depend in a large degree on the reduction of the losses associated with the process of concentration. It was therefore attempted to improve every phase in the preparation of the material. The starting point of our work was the fraction precipitable by 80 per cent alcohol and prepared in a general way according to directions of Osborne and Wakeman and referred to in this communication as the "O. W." fraction.[2] In the earlier work a quantity of this material, containing 3.0 mg. of nitrogen, was the minimum required to promote growth. This material was now thoroughly shaken with absolute alcohol until it acquired the character of a very fine powder which, on drying under reduced pressure, was no longer hygroscopic. The material prepared in this manner was active in quantities of from 10 to 15 mg. (containing 1.0 to 1.5 mg. of nitrogen) per day.

Precipitation with Basic Lead Acetate.

The first phase in the purification of this material in the earlier work consisted in precipitation by means of barium hydroxide. It was now found advantageous to add as an intermediary step the precipitation of the active substance by means of basic lead acetate. Lead acetate had been applied for this purpose on previous occa-

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[1] Levene, P. A., and van der Hoeven, B. J. C., *J. Biol. Chem.*, 1924, lxi, 429.

[2] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.* 1919, xl, 383.

sions, but unsuccessfully. The cause of the failure lay in the method which was employed for the liberation of the active material from the lead precipitate; at that time, hydrogen sulfide was used for the removal of the lead and no effective way was found to recover the active principle from the sulfide of lead.

It was now found that when the lead was removed by means of sulfuric acid, the active principle could be recovered practically quantitatively. By this step alone, the potency of the product increased three to four times.

To this material the precipitation with barium hydroxide was applied in the manner described in the first paper. By this treatment a product was obtained which was approximately as active as the one previously prepared through the silica adsorption· process. The new product was active in quantities containing from 0.10 to 0.20 mg. of nitrogen.; *i.e.*, in quantities of 2.0 to 4.0 mg. of the substance. The substance contained 5.2 per cent of nitrogen, calculated ash-free. From this it is seen that by the lead-barium precipitation a considerable quantity of inert nitrogenous material was removed, since this product contained less nitrogen than the starting material. The yield of this material was between 30 and 40 per cent.

Extraction with Silica Gel.

Further purification was again accomplished by extraction with silica gel and, as will be seen later, a selective purification was accomplished by this adsorbent. The adsorbed material was removed from the silica twice by alcohol, acidulated to pH 3. These extracts contained materials of only moderate potency. A third extraction was made at a pH of 9. This extract contained the most potent material thus far prepared. It was active in about 0.1 mg. of substance, containing 0.015 mg. of nitrogen. In view of the fact that the absolute volume of the solution and hence the proportions of acid and base used in the experiments were large in proportion to the material, it was thought advantageous to employ an acid of which the salts would be soluble in alcohol. Since it was found that the active principle is not soluble, it could thus be separated from the mineral impurities. With this aim in view, hydriodic acid was employed for the extraction of the silica. Nevertheless, the product obtained

from the silica extract by alcohol precipitation contained about 80 per cent of ash, though the material contained only traces of iodine. The substance dried in the desiccator (under diminished pressure) contained 3.1 per cent of nitrogen or 15 per cent calculated for the dry ash-free material. It contained 3.4 per cent of inorganic phosphorus and 4.6 per cent of total phosphorus. It gave negative tests for protein (Biuret and Millon's) and positive for pentoses. On hydrolysis the product reduced Fehling's solution. From all these data it is easily concluded that the substance was still of a very complex nature. The yield of this material was about 35 per cent of the lead and barium product, or between 3 and 5 per cent of the Osborne-Wakeman fraction.

Attempts to Remove the Mineral Constituents of the Active Principle.

The great disturbing fact in the purification of the growth-promoting principle by means of adsorbents is the coadsorption and even introduction of mineral impurities. Of these, the salts of phosphoric acid are particularly disturbing. These can be removed from the lead and barium product by precipitating the active material with absolute alcohol from strongly acid solution (pH 2). By precipitating under these conditions the sulfuric acid extract of the barium precipitate, a substance was obtained which was potent in quantities of about 2.5 mg. (containing 0.10 mg. of nitrogen) per day. This product contained only 6.1 per cent of mineral impurity. It contained no phosphorus. It had the following composition. C = 40.0 per cent; H = 5.8 per cent; N = 4.1 per cent. The substance gave a strong orcinol test and on hydrolysis reduced Fehling's solution. It is thus seen that in the main, this product consisted of carbohydrates. Whether the carbohydrate is an integral part of the active principle or is only an inert admixture cannot be said.

A material still more potent and apparently of the same composition was obtained when the lead-barium product containing the mineral impurities was dissolved in a minimal amount of hydrochloric acid of specific gravity 1.19 and to the solution enough 98 per cent alcohol was added to make the alcohol concentration 70 per cent. This material still contained about 4 per cent of nitrogen and was active in daily doses of 1.25 mg.

However, it was found that the material purified in this manner was not as readily further improved by silica adsorption as was the phosphorus-containing material. It was stated above that the product obtained through silica adsorption from the latter material was potent in daily doses of 0.10 mg. and contained 15 per cent, whereas by the same treatment of the purified product, a substance was obtained which contained only 4.2 per cent of nitrogen and which was active in doses of 0.6 mg. per day. Thus it seems as if the phosphorus-containing impurity is essential for the selective adsorption by silica.

EXPERIMENTAL PART.

Precipitation with Lead and Barium.

250 gm. of purified Osborne and Wakeman's fraction are dissolved in water, centrifugalized after one night's standing in a cold room, and precipitated with 5 liters of basic lead acetate solution. The precipitate collected by centrifugalization is treated with excess 10 per cent sulfuric acid until it reacts acid to Congo paper and is then centrifugalized. To the clear solution are added 8 liters of a cold saturated barium hydroxide solution sufficient to make it alkaline. The precipitate is rapidly centrifugalized and taken up in 10 per cent sulfuric acid. The filtrate from the barium sulfate is used for further purification.

Lead-Barium Precipitation:

O.W. Fraction.

(1.2)·   10 gm. N

|8000| units

Precipitated basic lead acetate.

Filtrate No. 145   Precipitate No. 146
(2.2)   3.8 gm. N   (0.3)   2.3 gm. N

= |1500| units   = |7000| units

Precipitated barium hydroxide.

Filtrate No. 147   Precipitate No. 148
(1)   1.6 gm. N   (0.15)   0.5 gm. N

= |1600| units   = |3300| units

O.W. Fraction.

(1.5)   3 gm. N

|2000| units

|— With basic lead acetate. —|

Filtrate No. 123   Precipitate No. 124
(4)   2.3 gm. N   (0.5)   1.00 gm. N

= |600| units   = |2000| units

|— With barium hydroxide. —|

Filtrate No. 125   Precipitate No. 131
(0.8)   0.81 gm. N   (0.15)   0.090 gm. N

= |1000| units   = |600| units

Extraction with Silica Gel.

A quantity of solution corresponding to 250 gm. of the O. W. fraction is brought to pH 5 and kept at that for ½ hour with continuous stirring and addition of 500 gm. of silica gel. The silica is then filtered off, suspended in 3 liters of 30 per cent alcohol, kept at pH 3 (with HI), filtered, this last process repeated once more, and finally the silica is suspended in 3 liters of distilled water, stirred, and kept at pH 9 for ½ hour. The clear solution obtained from this extraction is immediately neutralized and evaporated to a small volume, the silicic acid removed, and the substance (5 cc.) precipitated by 250 cc. of absolute alcohol.

Extraction with Silica Gel.

Lead-barium precipitate.
(0.15)   1.60 gm. N
|11,000| units

|— Extracted with 500 gm. silica at pH 5. —|

Filtrate No. 174   1.   Extract (pH 3) No. 175
(0.20)   1.31 gm. N   (0.09)   0.12 gm. N

= |6500| units   = |1300| units

2.   Extract (pH 3) No. 176
(0.08)   0.05 gm. N

= |600| units

3.   Extract (pH 9) No. 177
(0.015)   0.02 gm. N

= |1400| units

Experiments with Substances from Which the Phosphates Had Been Removed.

No. 393   (Lead-barium precipitate).

(0.17)   0.070 gm. N

|400| units

┌──┐
│   With absolute alcohol at pH 2.   │

Filtrate No. 394   Precipitate No. 395
(0.20)   0.045 gm. N   (0.10)   0.020 gm. N

= |220| units   = |200| units

No. 417   (Phosphate-free).

(0.12)   0.020 gm. N

|160| units

┌──┐
│   With HCl of sp. gr. 1.19, and 70 per cent alcohol. │

Filtrate No. 419   Precipitate No. 420
(0.15)   0.012 gm. N   (0.05)   0.0042 gm. N

= |80| units   = |80| units

No. 395 and following (Phosphate-free).

(0.15)   0.060 gm. N

|400| units

┌──┐
│   Extracted with 500 gm. silica gel at pH 5.   │

Filtrate No. 440   1.   Extract (pH 3) No. 441
(0.35)   0.050 gm. N   (Inactive)   0.002 mg. N

= |140| units |0|units

                               3.   Extract (pH 9) No. 442
                                   (0.025)   0.04 mg. N

                                   |160| units

Analytical Data.

Substance.	N in ash-free sub-stance.	C	H	Activity.	Qualitative reactions.
	per cent	*per cent*	*per cent*	*mg. N*	
Osborne-Wakeman fractions.........	10.1			1.0–1.5	
Lead-barium precipitate.............	5.2			0.10	
Silica extract from lead-barium pre-cipitate........................	15			0.015	Molisch + Millon − Biuret −
Lead-barium precipitate, phosphate-free.............................	4.1	40.0	5.8	0.10	Orcinol + Biuret − Fehling +
Silica extract from lead-barium pre-cipitate, phosphate-free...........	4.2			0.025	

CONCLUSIONS.

1. The Osborne and Wakeman concentrate of the yeast extract can be further concentrated.

2. By successive precipitation, first by lead acetate and second by barium hydroxide, a product is obtained which contains 5.2 per cent of nitrogen (calculated for the ash-free substance) and is potent in daily doses of from 2.0 to 4.0 mg. per day.

3. The ash-free product from the material described in (2) has an elementary composition approaching that of carbohydrates. It yields on hydrolysis reducing sugars, contains about 4 per cent of nitrogen, and is potent in daily doses of 1.25 mg. per day.

4. From the material described in (2) by silica adsorption a product is obtained which is potent in daily doses of 0.100 mg. and which contains 15 per cent of nitrogen.

5. From the product described in (3) by the silica treatment a material is obtained which is potent in daily doses of 0.600 mg. and which contains only 4 per cent of nitrogen.

STROPHANTHIN.

VIII. THE CARBONYL GROUP OF STROPHANTHIDIN.

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The presence of olefinic groups in dianhydrostrophanthidin has been demonstrated by previous hydrogenation experiments[1] and it was hoped that these double bonds might be made the point of attack in oxidation experiments with the formation of substances, either degradation products or those which would give information as to the position of the double bonds. However, as we have previously noted this substance exhibits towards permanganate in acetone solution no greater degree of unsaturation than strophanthidin itself. In agreement with this observation oxidizing agents as employed in the present experiments have given substances in which the double bonds have resisted oxidation and in which the carbonyl group instead has been the point of attack. But a study of these substances has permitted the drawing of definite conclusions regarding the nature of the carbonyl group in strophanthidin and its derivatives.

Although no positive evidence was previously available the carbonyl group has been referred to as probably ketonic in character. This was inferred mainly from the behavior of strophanthidin and its derivatives towards aldehyde reagents. Strophanthidin, dihydrostrophanthidin, and isostrophanthidin do not reduce Fehling's solution. Although strophanthidin itself reduces Tollens' reagent this property we now know is referable to the unsaturated lactone group since it is lost by conversion into the dihydro derivative. Likewise, isostrophanthidin only very slowly reduces this reagent. An aromatic aldehyde group seemed out of the question although a tertiary

[1] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiii, 123.

aliphatic aldehyde might still have permitted the above behavior. But in the deportment of these substances towards alkali, no positive evidence of a Cannizzaro reaction was obtained. By the action of alcoholic alkali it was possible to convert strophanthidin quantatively into isostrophanthidin although boiling alkali was found to give rise to obscure, highly colored, amorphous alteration products. The present studies, however, on the behavior of dianhydrostrophanthidin, strophanthidin, and dihydrostrophanthidin towards oxidizing agents, appear to demonstrate definitely the aldehydic character of the carbonyl group.

When dianhydrostrophanthidin was oxidized in acetic acid solution with chromic acid a neutral crystalline substance, $C_{23}H_{26}O_4$, was the principal reaction product which formed neither an oxime nor an acyl compound. Its behavior towards boiling alkali demonstrated it to be a dilactone. Both lactone groups were easily saponified to a dibasic acid, $C_{23}H_{30}O_6$, which showed little tendency to lactonize again. The dilactone still reduced Tollens' reagent, showing the retention of the original unsaturated lactone group. The new lactone group was formed presumably by direct oxidation of the oxidic form of dianhydrostrophanthidin since the dilactone when once opened showed little tendency to close again, at least under conditions which resembled those which obtained during the oxidation. However, a very small amount of an acid by-product was isolated from the reaction mixture which possessed the composition, $C_{23}H_{28}O_5$, and proved to be a lactone acid. The lactone group was that originally present in dianhydrostrophanthidin and the acid group was formed by direct oxidation of the aldehydic form. When saponified this acid gave the above described dibasic acid. In other words either dianhydrostrophanthidin in acetic acid solution exists as an equilibrium mixture between the oxidic and aldehydic forms in which the former apparently preponderates; or, as appears more likely, the chromic acid acts far more specifically upon the oxidic form so that the equilibrium of the mixture is constantly shifting to the oxidic form as the latter is disposed of by the oxidizing agent. Potassium permanganate was also found to exhibit a specific behavior towards dianhydrostrophanthidin in acetone solution. Although most of the substance was recovered unchanged the only crystalline

oxidation product which could be isolated was the above lactone acid in very small amount and in this case no evidence of the formation of the dilactone could be obtained. We shall note again similar instances of the specific behavior of these oxidizing agents.

The dilactone, $C_{23}H_{26}O_4$, in acetic acid solution was readily hydrogenated by palladium and hydrogen. Little difficulty was experienced with the hydrogenation of the two double bonds which, as we shall see, are apparently associated with the new lactone group, but the saturation of the third olefinic linking, and that originally present in strophanthidin, proved to be just as refractory as in the case of strophanthidin itself. Only by the use of a pure solvent and a very active palladium was it possible to introduce the third mol of hydrogen. In the first case the resulting tetrahydrodilactone, $C_{23}H_{30}O_4$, although possessing no free carboxyl or hydroxyl groups still showed the presence of one active hydrogen. The hexahydrodilactone no longer gave methane with Grignard reagent. The active hydrogen of the tetrahydro compound must belong to the α-methylene situated between the lactone CO group and the β-γ double bond. This property is lost on further hydrogenation.

$$-\overset{|}{C} = \overset{|}{C} - \overset{*}{CH_2} - CO$$
$$\underline{\hspace{1cm}O\hspace{1cm}}$$
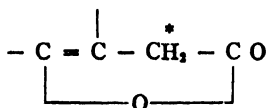
In support of this is the behavior of these substances towards Tollens' reagent. The tetrahydrodilactone reduces the reagent whereas the hexahydro compound is without action upon it.

The tetrahydro compound proved to be of special interest in the behavior of its lactone groups towards alkali. Whereas the original unhydrogenated dilactone was quantitatively saponified by the usual 2 hours' boiling in 0.1 N sodium hydroxide solution, under these conditions only one group was saponified in the case of the tetrahydrodilactone. It was finally ascertained that this refractoriness was not absolute but only relative and that after 6 hours' boiling in normal sodium hydroxide solution the second lactone group could be completely opened. Similar observations were made with the hexahydrodilactone and in this case it was possible to ascertain, as seemed *a priori* likely, that the refractory lactone group was that

associated with the tetrahydrogenated double bonds. When only one lactone group was opened with a weak alkali, reacidification resulted in the recovery of the original hexahydrodilactone. Under the same conditions the tetrahydrodilactone yielded a lactone acid. This conclusion is supported by the fact that the dihydrogenated lactone group of dihydrostrophanthidin and its derivatives, as previously noted, relactonizes very readily after saponification.[2]

In view of the oxidation of the aldehydic group of dianhydrostrophanthidin to the lactone acid we have returned to a consideration of our former experience with strophanthidin itself.[3] This substance in acetone solution had yielded with permanganate a substance which was described as a lactone acid, $C_{23}H_{30}O_7$, in which in some apparently obscure way the carbonyl group had been changed to carboxyl with simultaneous loss of two H atoms. This formula has now been shown to be incorrect and the acid there described possesses instead the formula $C_{23}H_{32}O_7$. The former uncertainty was largely due to the difficulty of drying without decomposition for analysis the substance which crystallized with water of crystallization. It is formed by the oxidation of the aldehydic group of strophanthidin to carboxyl. This was substantiated by the formation of the analogous dihydrolactone acid, $C_{23}H_{34}O_7$, by oxidation of dihydrostrophanthidin with permanganate both in acetone solution and after saponification in aqueous solution. This last procedure was inapplicable in the case of strophanthidin itself because of isomerization to isostrophanthidin. With the idea of showing the relationship of these acids to those obtained from dianhydrostrophanthidin an attempt was made to dehydrate them with concentrated hydrochloric acid. It was surprising, however, that instead of the expected anhydrolactone acids or perhaps anhydrodilactones neutral substances were obtained which differed from the original acids by only 1 mol of water. In other words the acids, $C_{23}H_{32}O_7$ and $C_{23}H_{34}O_7$, obtained respectively from strophanthidin and dihydrostrophanthidin, were converted into the neutral dilactones, $C_{23}H_{30}O_6$ and $C_{23}H_{32}O_6$. The former retained the power to reduce Tollens' reagent and the latter did not. These dilactones curiously enough exhibited a

[2] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiv, 383.
[3] Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 556.

behavior towards alkali similar to that shown by the tetrahydro-
and hexahydrodianhydrodilactones in that the newly formed lac-
tone group proved to be much more resistant to alkali than that
originally present.

In view of the comparative rigidity of this second lactone or
oxidic structure in the saturated substances a clue was obtained as
to the nature of the so called pseudostrophanthidin described in a
previous communication[4] and which was formed by the action of
strong hydrochloric acid on strophanthidin. This appeared to
represent a stable oxidic form of the hydroxyaldehyde since it did
not form an oxime. The unsaturated lactone group was demons-
trated to be still intact by its behavior towards Tollens' reagent.
Definite proof of the oxidic form was obtained by oxidation. Al-
though permanganate in acetone solution was practically without
action upon pseudostrophanthidin it was easily oxidized by chromic
acid. Instead, however, of the expected dilactone, $C_{23}H_{30}O_6$, identical
with that described above, a neutral substance, $C_{23}H_{28}O_6$, was ob-
tained which was also found to contain two lactone groups. Further
inquiry showed that the oxidation had simply proceeded a step
further with oxidation apparently of one of the hitherto dormant
hydroxyl groups to a ketone. The same ketodilactone was obtained
by further oxidation with chromic acid of the above dilactone,
$C_{23}H_{30}O_6$, obtained from strophanthidin.

The experience has thus shown rather a sharp contrast between
the behavior of permanganate and chromic acid as oxidizing agents.
The former appears to have acted specifically by oxidizing directly
the aldehydic form to the acid. The previously described oxida-
tion of isostrophanthidin or at least of isostrophanthidic acid to
isostrophanthic acid with permanganate belongs unquestionably in
this category. On the other hand, chromic acid appears to be
specific for the conversion of the oxidic form of the aldehyde into
the lactone.

Although the experience given here permits the coordination of
a number of observations which have been made in the study of
strophanthidin and its derivatives a number of uncertainties still

[4] Jacobs and Collins,[1] p. 131.

persist. Such questions as the bearing of the rigidity of the satu-
rated oxidic forms on structure and the reason for the failure of
pseudostrophanthidin, and the lactone acid, $C_{23}H_{32}O_7$, and other
derivatives which still possess the unsaturated lactone ring of stro-
phanthidin, to isomerize to iso compounds under the influence of
alkali remain to be answered. Again, the apparent failure of the
acid, $C_{23}H_{32}O_7$ (then erroneously described as $C_{23}H_{30}O_7$), to react
with hydroxylamine after saponification will require further study.
These and other questions are at present under investigation.

Since pseudostrophanthidin and the dilactone, $C_{23}H_{30}O_6$, yield a
ketodilactone, it would seem that at least one of the hydroxyl groups
originally present must be secondary. Since in all likelihood the
saturated lactone group in this compound is γ-oxidic and the same
hydroxyl is presumably involved in the ring closure as that which
is concerned in the oxidic forms of the anhydrostrophanthidins and
which in the aldehydic form can be acylated, doubt is cast upon the
previous interpretation which attributed to the remaining hydroxyls
of strophanthidin a tertiary character. This conclusion was origin-
ally based on the failure to acylate more than one hydroxyl group.
The possibility now appears that strophanthidin may have at least
two free secondary hydroxyl groups. Further work may demon-
strate all of them to be secondary, in which case strophanthidin will
begin to assume the appearance of a complex molecule with a carbo-
hydrate side chain. Such speculations must, however, await the
accumulation of further data. Interesting in connection with the
observations with these substances is the fact that all of the neutral
hydroxy compounds still possess an appreciably bitter taste although
not quite as marked as in the case of strophanthidin. These sub-
stances may also exhibit interesting physiological behaviors. This
study is contemplated.

EXPERIMENTAL.

Oxidation of Dihydrostrophanthidin and Strophanthidin.

Lactone Acid, $C_{23}H_{34}O_7$.—3 gm. of dihydrostrophanthidin were
shaken at ordinary temperature in 30 cc. of N sodium hydroxide
solution. Solution was complete within 10 minutes. After diluting
to 100 cc. the mixture was oxidized at 10° with 17.5 cc. of 5 per cent

permanganate or an amount slightly in excess of that required for 1 mol of O. The reagent was at first fairly promptly consumed, but towards the end the permanganate color persisted for some time. The filtrate was carefully acidified to Congo red with hydrochloric acid and, on rubbing, the lactone acid gradually separated as a sandy powder which consisted of small prisms and stout, pointed platelets. The mother liquor yielded an additional amount when concentrated at room temperature. It was found, however, advisable to neutralize the solution before concentration and then to reacidify to Congo red since the acid proved to be sensitive to a too vigorous treatment with mineral acid and was readily converted into amorphous material (partial lactonization). The total yield was 2.2 gm. The acid when recrystallized by careful dilution of a concentrated alcoholic solution slowly separated as a crust of short, stout platelets which contained 2 mols of water of crystallization. It slowly froths up when heated to 132–133° and is easily soluble in alcohol and acetone and not appreciably soluble in the water-immiscible solvents. In sulfuric acid it forms at first a brown-orange color which deepens to a very characteristic deep purple-red.

$$[\alpha]_D^{22} = +47° \ (c = 1.015 \text{ in methyl alcohol for the hydrate}).$$

During the drying of the substance for analysis it appeared that slight decomposition occurred since the carbon figures were always too high. This recalls the experience with the analogous acid obtained from strophanthidin and also the experience with strophanthidin itself.

Air-Dry Substance.   Dried at 100° and 15 mm. over H_2SO_4.
                    $C_{21}H_{34}O_7 \cdot 2H_2O$. Calculated. H_2O 7.86.
                                Found.   " 7.88.
Anhydrous Substance.
                    $C_{21}H_{34}O_7$. Calculated. C 65.36, H 8.12.
                                Found.   " 65.95, " 8.23.

If in the above oxidation experiment the addition of permanganate sufficient to furnish 1 mol of oxygen was followed by the further addition of four times that amount the permanganate was very slowly consumed. From the reaction mixture it was still possible to isolate a small amount of the above acid and no evidence was obtained of

the formation of a ketonic acid analogous to that previously obtained by the oxidation after saponification of the lactone acid obtained from strophanthidin.[5]

The lactone acid with identical properties was also prepared although less conveniently by the use of permanganate in acetone solution in accordance with the procedure previously described for the preparation of the lactone acid from strophanthidin.

Air-Dry Substance. Found. H_2O 8.49, 8.19.
Anhydrous Substance. Found. (*a*). C 65.75, H 8.26.
                                (*b*). " 65.93, " 7.99.

0.1049 gm. of substance when titrated with 0.1 N NaOH against phenolphthalein required 2.30 cc. Calculated for 1 equivalent, 2.48 cc. After further addition of an excess of alkali the mixture was refluxed for 2 hours and again titrated. An additional 2.52 cc. were consumed.

Dilactone, $C_{23}H_{32}O_6$.—1.1 gm. of the above lactone acid were dissolved in 10 cc. of concentrated hydrochloric acid and the solution was kept in ice for 20 minutes during which it deepened to an olive color. Dilution with water caused the precipitation in small amount of yellow amorphous material, leaving a colorless solution after filtration. On standing the dilactone slowly crystallized as a sandy powder consisting of rosettes of microplatelets. When recrystallized from dilute alcohol it formed lustrous leaflets which contained 0.5 mol of water of crystallization. The substance melts at 232–234° after preliminary softening and is insoluble in cold dilute alkali. It is easily soluble in acetone and chloroform and less readily so in alcohol. In sulfuric acid it gives on standing the characteristic red-purple color of the original acid. In dilute pyridine solution it does not reduce Tollens' solution. The substance possesses an appreciably bitter taste.

$$[\alpha]_D^{28} = +84° \ (c = 1.000 \text{ in methyl alcohol}).$$

The two lactone groups exhibited a marked difference in their resistance to the saponifying action of alkalies which was shown

[5] Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 559.

in the following titration experiments. 0.0618 gm. of anhydrous substance was boiled for 2 hours in 15 cc. of alcohol and 15 cc. of 0.1 N NaOH and titrated against phenolphthalein. Calculated for 1 equivalent, 1.53 cc. Found, 1.89 cc. 0.1085 gm. of substance was boiled for 3 hours in 15 cc. of 0.2 N NaOH. Calculated for 2 equivalents, 2.68 cc. Found, 2.24 cc. For complete saponification even longer boiling was apparently necessary.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.
$$C_{23}H_{32}O_6 \cdot \tfrac{1}{2}H_2O. \text{ Calculated. } H_2O \text{ 2.18.}$$
$$\text{Found. } \text{" 1.98.}$$

Anhydrous Substance.
$$C_{23}H_{32}O_6. \text{ Calculated. } \text{C 68.27, H 7.98.}$$
$$\text{Found. } \text{" 68.36, " 7.95.}$$

Dilactone, $C_{23}H_{30}O_6$.—1 gm. of the lactone acid, $C_{23}H_{32}O_7$, which was obtained by oxidation of strophanthidin with permanganate in acetone solution, and which, in a previous communication,[6] was given the incorrect formula, $C_{23}H_{30}O_7$, was converted into the dilactone in accordance with the procedure used above in the case of the dihydrolactone acid. After several days, crystallization began in the diluted reaction mixture. The separation was facilitated by salting-out with ammonium sulfate. Recrystallized from dilute alcohol it forms leaflets and long pointed platelets containing approximately 1 mol of water and is insoluble in alkali. It melts at 235–236° after preliminary sintering and, in sulfuric acid, gives at first an orange-brown color which changes to the characteristic purple-red color given by the original acid. It is soluble in acetone, chloroform, and alcohol. In dilute pyridine solution it reduces Tollens' solution.

$$[\alpha]_D^{27} = +100° \ (c = 1.005 \text{ in methyl alcohol}).$$
Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.
$$C_{23}H_{30}O_6 \cdot H_2O. \text{ Calculated. } H_2O \text{ 4.28.}$$
$$\text{Found. } \text{" 3.38.}$$

Anhydrous Substance.
$$C_{23}H_{30}O_6. \text{ Calculated. } \text{C 68.61, H 7.52.}$$
$$\text{Found. } \text{" 69.04, " 7.62.}$$

0.0931 gm. of substance was boiled for 5 hours at 15 cc. of 0.2 N

[6] Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 556.

NaOH and titrated against phenolphthalein. Calculated for 2 equivalents, 2.32 cc. Found, 2.15 cc.

Ketodilactone, $C_{23}H_{28}O_6$.—A solution of 1 gm. of pseudostrophanthidin in 5 cc. of acetic acid was treated with 2 cc. of chromic acid solution which was prepared by mixing 400 gm. of water, 80 gm. of sulfuric acid, and 53 gm. of chromium trioxide.[7] The reaction was kept under control by cooling and after completion the mixture was diluted with water. Lustrous leaflets separated on rubbing. The substance was most conveniently recrystallized by dissolving in a necessarily large volume of hot alcohol or acetone, and concentrating to crystallization. It formed small rhombic and wedge-shaped prisms which melted with effervescence at 285° after preliminary softening, and were insoluble in cold alkali. The solution in·sulfuric acid gradually developed an amber color.

$$[\alpha]_D^{18} = + 93° (c = 1.01 \text{ in pyridine}).$$
$$C_{23}H_{28}O_6. \text{ Calculated. } \text{C } 68.97, \text{ H } 7.05.$$
$$\text{Found. } " 69.03, " 7.09.$$

The presence of two lactone groups was shown as follows: 0.1226 gm. of substance was refluxed for 5 hours with 15 cc. of 0.2 N NaOH and titrated against phenolphthalein. Calculated for 2 equivalents, 3.06 cc. Found, 3.00 cc. The identical substance was obtained by replacing in the above oxidation experiment pseudostrophanthidin by the dilactone, $C_{23}H_{30}O_6$. The resulting substance possessed the same properties and melting point as the above oxidation product and a mixed melting point showed no depression.

$$[\alpha]_D^{28} = + 97° (c = 1.01 \text{ in pyridine}).$$
$$\text{Found. C } 68.73, \text{ H } 6.92.$$

Oxime of the Ketodilactone.—The above oxidation product was refluxed for 2 hours in 20 parts of alcohol with hydroxylamine hydrochloride and sodium acetate. During the reaction the oxime separated incompletely. After collecting it was washed free of salt with water. The mother liquor yielded additional amounts when concentrated. It was recrystallized by dissolving in a necessarily large volume of hot alcohol and by subsequent concentration to crystalliza-

[7] Kiliani, H., *Ber. chem. Ges.*, 1913, xlvi, 676.

tion.   The oxime formed a sandy deposit of minute rhombs and short, stout prisms which melted with effervescence above 285°.

$$C_{23}H_{29}O_4N. \text{Calculated. C 66.46, H 7.04.}$$
$$\text{Found.   `` 66.51, `` 6.90.}$$

Oxidation of Dianhydrostrophanthidin.

Dilactone, $C_{23}H_{26}O_4$.—10 gm. of dianhydrostrophanthidin were dissolved in 200 cc. of acetic acid.   With cooling the mixture was treated at once with 35 cc. of a Kiliani chromic acid solution.[7]   The mixture was warmed and there was appreciable gas evolution.   After 10 minutes the solution was poured into water.   The resulting copious voluminous precipitate rapidly crystallized.   After collecting the precipitate with water it was digested with dilute ammonia in order to dissolve small amounts of acid by-products.   The ammoniacal extract yielded very small amounts of an acid which will be described below.   The neutral precipitate after collecting was dissolved in a necessarily large volume of hot dry acetone and the solution was concentrated to smaller bulk.   The dilactone crystallized as lustrous, flat needles and six-sided plates which melted at 253–254° after preliminary softening.   By working up the mother liquor the yield obtained was 4.2 gm.   The substance is readily soluble in chloroform and hot acetic acid, appreciably soluble in acetone, and but sparingly so in alcohol.   In sulfuric acid it gives at first a yellowish brown solution which deepens to a deep brown-red and on standing to a deep purple.   In dilute pyridine solution it reduces Tollens' reagent due to the unsaturated lactone ring which has resisted the oxidation.

$$[\alpha]_D^{25} = -178° (c = 1.000 \text{ in chloroform}).$$
$$C_{23}H_{26}O_4. \text{Calculated. C 75.37, H 7.16.}$$
$$\text{Found.   `` 75.49, `` 6.94.}$$

Both of the lactone groups were readily saponified and titrated as follows: 0.1013 gm. of substance was refluxed for 2 hours in a mixture of 20 cc. of alcohol and 20 cc. of 0.1 N NaOH and titrated back against phenolphthalein.   Calculated for 2 equivalents, 5.54 cc.   Found, 5.7 cc.

With Grignard reagent the substance still shows an active hydrogen atom which is probably associated with the α-carbon atom adjoining

the carbonyl and the β-unsaturated carbon atom of the Δ β-γ-crotonic lactone group.

0.0467 gm. substance: 2.72 cc. CH_4 (0°, 760 mm.) or 0.94 mol for mol. wt. 366.

Lactone Acid, $C_{23}H_{28}O_5$.—The above described ammoniacal extract of the crude oxidation product when acidified with hydrochloric acid gave a very small amount of amorphous precipitate. The combined material from a number of experiments was boiled up with acetone and the filtrate concentrated to small volume. Rectangular platelets separated which melted with effervescence at 268°.

$$[\alpha]_D^{25} = -100° \ (c = 1.025 \text{ in } 95 \text{ per cent alcohol}).$$
$$C_{23}H_{28}O_5. \quad \text{Calculated.} \quad C \ 71.84, H \ 7.35.$$
$$\text{Found.} \quad \text{" } 71.60, \text{ " } 7.37.$$

This acid was also obtained in very small amount by extracting the dilute acetic acid filtrate from the crude oxidation product with chloroform. When shaken out with dilute ammonia the monobasic lactone acid was obtained after acidification. The same acid was obtained as follows when dianhydrostrophanthidin was oxidized in acetone solution with permanganate and no evidence of the formation of the dilactone was obtained: 5 gm. of dianhydrostrophanthidin were dissolved in 300 cc. of hot dry acetone. The solution was cooled to room temperature and then shaken with 3 gm. of potassium permanganate until decolorized, a process which took about 15 minutes. The collected precipitate was extracted with water and the filtrate was acidified with acetic acid. The very small amount of crude acid crystallized from dilute acetone as six-sided leaflets which contained 1 mol of water and melted with effervescence at 272–274°. It showed no depression when mixed with the lactone acid obtained above as a by-product in the chromic acid oxidation.

$$[\alpha]_D^{25} = -102° \ (c = 1.025 \text{ in } 95 \text{ per cent alcohol}).$$

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4.
$$C_{23}H_{28}O_5 \cdot H_2O. \quad \text{Calculated.} \quad H_2O \ 4.47.$$
$$\text{Found.} \quad \text{" } 4.13.$$

Anhydrous Substance.
$$C_{23}H_{28}O_5. \quad \text{Calculated.} \quad C \ 71.84, H \ 7.35.$$
$$\text{Found.} \quad \text{" } 71.16, \text{ " } 7.33.$$

0.1076 gm. of anhydrous substance when titrated directly against

phenolphthalein required 2.9 cc. of 0.1 N NaOH. After the addition of an excess of alkali with subsequent boiling for 2 hours an additional 3.1 cc. were consumed. Calculated for 1 equivalent, 2.8 cc. When this saponification mixture was acidified with acetic acid and the resulting acid was recrystallized from 50 per cent acetic acid it formed fine needles which melted with effervescence at 242–243° and showed no depression when mixed with the dibasic acid which was obtained from the dilactone as follows:

Dibasic Acid, $C_{23}H_{30}O_6$.—The dilactone was saponified as previously described. After neutralization the alcohol was removed under diminished pressure and the mixture was then acidified. The partly crystalline precipitate after collection was recrystallized from 50 per cent acetic acid. It formed needles which melted with effervescence at 249–251° although frequently a melting point of 242–243° was observed. The method of recrystallization employed shows the slight tendency to lactonize possessed by the substance. 0.1052 gm. of substance when titrated against phenolphthalein required 5.00 cc. of 0.1 N NaOH. Calculated, 5.24 cc.

$C_{23}H_{30}O_6$. Calculated. C 68.61, H 7.52.
Found. " 69.04, " 7.43.

Tetrahydrodilactone, $C_{23}H_{30}O_4$.—4 gm. of dilactone were dissolved in about 100 cc. of pure glacial acetic acid and reduced with 0.8 gm. of palladium black and hydrogen. The first mol was absorbed within the first 2 hours and after 24 hours the second mol of hydrogen was used up. The solution when poured into water yielded the crystalline hydrogenation product. When recrystallized from alcohol 2.5 gm. of delicate needles were obtained which melted at 275–277° with slight preliminary sintering. The mother liquor on concentration yielded lower melting material which was not further investigated and which possibly consisted of stereoisomers. The presence of the unsaturated lactone group was shown by the fact that in dilute pyridine solution it slowly reduced Tollens' reagent. The solubility of the substance decreases in the order: chloroform, acetone, alcohol, and benzene.

In sulfuric acid it gives a yellow color.

$[\alpha]_D^{26} = + 3.0°$ ($c = 1.000$ in chloroform).
$C_{23}H_{30}O_4$. Calculated. C 74.54, H 8.17.
　　　　　　Found. " 74.37, " 8.05.

The marked difference in stability of the two lactone groups towards alkali was demonstrated by the following saponification experiments. 0.1109 gm. of substance was refluxed for 2 hours in a mixture of 15 cc. of alcohol and 15 cc. of 0.1 N NaOH and titrated against phenolphthalein. Found, 3.07 cc. Calculated for 1 equivalent, 3.00 cc.

When the saponification mixture was acidified to Congo red a partly crystalline precipitate formed. After collecting with water it was suspended in 50 per cent alcohol and dissolved by addition of ammonia. There remained a small amount of apparently neutral crystalline residue, formed probably by relactonization. The filtrate on acidification yielded microscopic bundles of lens-shaped leaflets of the lactone acid which sintered above 215° and slowly melted at 225–230°.

Both lactone groups, however, were saponified under the following conditions. 0.5263 gm. of substance was refluxed for 5 hours in a mixture of 10 cc. of N NaOH and 5 cc. of alcohol. Found, 2.68 cc. Calculated for 2 equivalents, 2.84 cc. When this mixture was acidified a voluminous amorphous precipitate formed which was collected with water. The alcoholic solution was carefully treated with water to incipient turbidity and globular masses slowly separated which were collected with dilute alcohol. The dibasic acid of questionable purity melted at 215° but the amount available made its further study unprofitable. 0.0516 gm. of this substance required 2.10 cc. of 0.1 N NaOH for neutralization. Calculated for 2 equivalents, 2.54 cc.

Hexahydrodilactone, $C_{23}H_{32}O_4$.—After the absorption of the first 2 mols of hydrogen it was found in a number of experiments that further absorption did not occur. This recalls the difficulties experienced with strophanthidin itself. However, when the dilactone was carefully purified and a palladium black was prepared under very careful conditions it was found possible to carry the absorption into the 3 mol stage which, however, required several days for completion. When the reaction mixture was poured into water the

hydrogenation product crystallized. After repeated recrystalliza-
tion from alcohol it formed needles which after slight preliminary
sintering melted at 265–267° to a turbid liquid which cleared a few
degrees higher. It is easily soluble in chloroform and but sparingly
so in the other usual solvents.

The solution in sulfuric acid remains practically colorless. In
dilute pyridine solution it does not reduce Tollens' reagent.

$$[\alpha]_D^{25} = + 14° \ (c = 1.015 \text{ in chloroform}).$$
$$C_{23}H_{32}O_4. \quad \text{Calculated.} \quad \text{C } 74.14, \text{ H } 8.67.$$
$$\text{Found.} \qquad \text{" } 74.38, \text{ " } 8.79.$$

Boiling for 2 hours with 0.1 N NaOH resulted in saponification of
only one lactone group. 0.1002 gm. of substance was boiled in a
mixture of 20 cc. of alcohol and 20 cc. of 0.1 N NaOH. Found,
2.74 cc. Calculated for 1 equivalent, 2.69 cc.

When the saponification mixture was reacidified to Congo red
needles of the original dilactone quickly separated as shown by
melting point and other properties.

SUBSTITUTION BY HALOGEN OF THE HYDROXYL IN SECONDARY ALCOHOLS.*

BY P. A. LEVENE AND L. A. MIKESKA.

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It is well recognized that the outcome of substitution on the asymmetric carbon atom of an optically active substance is determined by the structure and by the polarity of the groups attached to the asymmetric carbon atom, on the one hand, and by the nature of the reacting substance, on the other.

In connection with secondary alcohols, a systematic study of the influences of the groups attached to the asymmetric carbon atom on the outcome of substitution is still missing. Pickard and Kenyon[1] have investigated the behavior of secondary normal alcohols. They observed that halogenation with hydrogen halides brought about a change in the direction of the rotation. McKenzie and Clough have observed that the action of thionyl chloride on methylphenyl carbinol is not accompanied with a change of direction of rotation. More recently Levene and Mikeska[2] and McKenzie and Tudhope[3] have found that the action of thionyl chloride on methylhexyl carbinol caused a change in the direction of rotation.

Thus, up to date, only two alcohols have been tested in regard to differences in the effect of the action of different halogenating agents. In one of the alcohols besides the methyl, a normal hexyl group is attached to the asymmetric carbon atom; in the other, a phenyl group. In the former, there was observed no difference in the effect of the two agents. In the latter, each of the reagents produced a

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* This is the fifth paper of the series on Walden inversion.

[1] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 45; *Ber. chem. Ges.*, 1912, xlv, 1592.

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473.

[3] McKenzie, A., and Tudhope, T. M. A., *J. Biol. Chem.*, 1924, lxii, 551.

chloride, one enantiomorphous to the other.   Hence, in one of two chlorides a Walden inversion took place. The question which now needs an answer is in which of the two the inversion occurred.

We now undertook the study of secondary alcohols in which the groups attached to the asymmetric carbon atom had a different character from the two described above. In one, isobutylmethyl carbinol, the second radicle attached to the asymmetric carbon atom contained a secondary carbon atom.

$$- CH_2CH \overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{\Big\langle}}$$
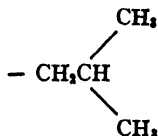
In the other, benzylphenyl carbinol, the two radicles attached to the asymmetric carbon atom contained a benzene ring. It differed from methylphenyl carbinol only in that 1 hydrogen atom in the methyl group of the latter was substituted by a phenyl group.

$$C_6H_5 - \overset{\displaystyle H}{\underset{\displaystyle OH}{C}} - CH_3 \longrightarrow C_6H_5 - \overset{\displaystyle H}{\underset{\displaystyle OH}{C}} - CH_2C_6H_5$$
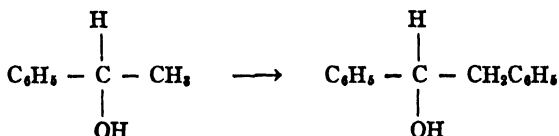
Besides, an additional representative of the normal secondary alcohol series, ethylmethyl carbinol, was tested in respect to its behavior towards thionyl chloride and other halogenating agents.

In each of the three alcohols halogenation was accomplished in two ways. In two, by means of hydrogen iodide and thionyl chloride, in the third by means of phosphorus pentachloride and of thionyl chloride. The action of hydrogen chloride with regard to the resulting direction of rotation is the same as that of hydrogen iodide.

Some interesting peculiarities in the behavior of individual alcohols came to light in the course of this work. In the two alcohols previously tested the chlorination with thionyl chloride proceeded without difficulty by carefully adding the alcohol to the thionyl chloride. The reaction proceeded equally readily in the case of benzylphenyl carbinol but, on the contrary, in the cases of ethylmethyl carbinol and of isobutylmethyl carbinol, thionyl chloride,

under these conditions, failed to react. Chlorination was brought about only by the method of Darzeus in pyridine solution. Besides, special conditions of temperature and duration of reaction had to be observed in order to avoid racemization. From these observations it seems suggestive that substitutions on the asymmetric carbon atom which proceed readily are accompanied with less racemization than those which take place with difficulty (slowly).

Resolution of Isobutylmethyl Carbinol.—The alcohol used in this experiment was prepared by Grignard's method from isovaleraldehyde and methyl iodide. To resolve the alcohol the general method of Pickard and Kenyon[4] was employed. The alcohol was converted into a half ester of phthalic acid by heating the molecular proportions of the alcohol and phthalic anhydride at 115°C. for 16 hours on the steam bath under a return condenser. The phthalate was converted into the brucine salt by treating it with one equivalent of brucine in acetone solution. On cooling, the brucinate precipitated. Much difficulty was experienced in separating the dextro from the levo component. If the crystallization was allowed to proceed too long, both forms came down. If interrupted in time, the dextro component was obtained. After many recrystallizations the brucinate was decomposed with hydrochloric acid and the phthalate was isolated by extraction with chloroform. After drying over sodium sulfate, the chloroform was removed. The residue showed a rotation of $[\alpha]^{20} = + 51.80°$.

Isobutylmethyl Carbinol.—55 gm. of the above phthalate were steam-distilled with $2\frac{1}{2}$ molecules (25 gm.) of sodium hydroxide. The alcohol when dried weighed 22 gm. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+ 0.98° \times 100}{1 \times 4.3821} = + 22.38°$$

Isobutylmethyl Iodide.—22 gm. of the carbinol obtained in the above experiment were slowly distilled with 80 gm. of hydriodic acid (b.p. = 126°). The iodide obtained was separated by means of a separatory funnel and distilled once more with 80 gm. of hydriodic acid. It was again separated from the aqueous layer, shaken out first with a little dilute sodium hydroxide, then with water, and

[4] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1907, xci, 2058.

finally dried over sodium sulfate. The iodide obtained weighed 22 gm. and showed a rotation of

$$[\alpha]_D^{20} = \frac{-1.24° \times 100}{1 \times 6.855} = -18.08°$$

2-Chloroisohexane.—10 gm. of isobutylmethyl carbinol, the rotation of which was $[\alpha]_D^{20} = -7.63°$, were mixed with 7.2 gm. of pyridine and then treated with thionyl chloride with just sufficient cooling to keep the temperature below 50°C. The mixture was then allowed to stand at 50° for 3 hours. It was then poured into water, extracted with ether, washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was removed and the residue fractionated:

FI......b.p. up to 115°C.   Weight = 4 gm.

$$[\alpha]_D^{20} = \frac{+2.74° \times 100}{1 \times 21.4} = +12.80°$$

This was combined with the corresponding fraction obtained in another experiment, and the two were redistilled.

FI'......b.p. up to 108°C.
FII'......" 108-114°.

For FII'.

$$[\alpha]_D^{20} = \frac{+3.68° \times 100}{1 \times 18.025} = +20.41°$$

0.1108 gm. substance: 0.1080 gm. AgCl.
$C_6H_{13}Cl$.   Calculated. Cl 29.42.
                Found.   " 24.11.

Resolution of Benzylphenyl Carbinol.—150 gm. of benzylphenyl carbinol were heated with 118 gm. of phthalic anhydride at 120° for 16 hours. The reaction mixture was then treated with two equivalents of sodium carbonate in aqueous solution and allowed to stand at room temperature for 1½ hours to decompose the unchanged phthalic anhydride. The unchanged carbinol was then extracted with ether and the residue acidified, whereupon the phthalate separated as an oil. It was extracted with chloroform, dried over sodium sulfate to precipitate the phthalic acid, and finally filtered from the phthalic acid and sodium sulfate. The chloroform was removed under

reduced pressure. The residue crystallized on standing. 220 gm. of the crude phthalate were obtained. It was dissolved in ether and reprecipitated in fine crystalline form with petrolic ether. The phthalate was then dissolved in acetone, treated with a little less than one equivalent of pure quinine, then more quinine was added until the solution showed a slightly alkaline reaction. On cooling, the quinine salt crystallized out. The solution was filtered and the salt extracted with hot acetone to dissolve as much as possible of the levo component. It was then recrystallized several times from 95 per cent alcohol. A fraction was finally obtained which on decomposition gave a phthalate with a rotation of

$$[\alpha]_D^{20} = \frac{+ 1.75° \times 100}{1 \times 6.978} = + 25.08°$$

The phthalate melted at 123°C.

The levo salt could be obtained from the recrystallization mother liquors which, when decomposed, gave a phthalate with a rotation of over $[\alpha]_D^{20} = -20°$.

d-Benzylphenyl Carbinol.—100 gm. of the half ester of *d*-benzylphenyl carbinol and phthalic acid ($[\alpha]_D^{20} = + 25.08°$) were treated with 40 gm. of sodium hydroxide in aqueous solution. The mixture was heated to boiling for about 10 minutes. It was then cooled and the carbinol, which separated as an oil and solidified on cooling, was extracted with ether. The extract was washed with water and dried over sodium sulfate. The solution was concentrated to a small volume and then treated with petrolic ether. The carbinol crystallized on standing. The first crop of crystals weighed 35 gm. 8 more gm. were obtained from the filtrates. Some of the first fraction was dissolved in ether and reprecipitated with petrolic ether. In this state of purity the substance melted at 64°C. and showed a rotation of

$$[\alpha]^{20} = \frac{+ 1.68° \times 100}{1 \times 9.060} = + 18.54°$$

d-Benzylphenyl Chloromethane (by Means of Thionyl Chloride).—10 gm. of *l*-benzylphenyl carbinol

$$[\alpha]_D^{20} = \frac{- 0.92° \times 100}{1 \times 9.837} = - 9.35°$$

were added in small amounts to 5 mols of thionyl chloride with cooling. The reaction mixture was then heated under a return condenser on a steam bath for 20 minutes. The excess of thionyl chloride was then removed under reduced pressure at 50–60°C. The residue was dissolved in ether, washed first with water, then with dilute sodium hydroxide, and finally with water again. The ether was removed under reduced pressure of 50°C. If higher temperature were used, the chloride decomposed into stilbene and hydrochloric acid. On cooling below zero the chloride solidified. Its exact melting point, however, was not determined. Without any further treatment the chloride was found to be analytically pure, as is evidenced from the analysis below.

0.1550 gm. substance: 0.1028 gm. AgCl.
$C_{14}H_{13}Cl$.   Calculated.   C 16.38.
Found.   " 16.40.

It had an optical rotation of

$$[\alpha]_D^{20} = \frac{+ 0.96° \times 100}{1 \times 13.036} = + 7.36°$$

l-Benzylphenyl Chloromethane (by Means of Phosphorus Pentachloride).—10 gm. of *d*-benzylphenyl carbinol having a rotation of

$$[\alpha]_D^{20} = \frac{+ 1.11° \times 100}{1 \times 6.631} = + 16.73°$$

were dissolved in 20 cc. of chloroform. This solution was added slowly with cooling to a suspension of 14 gm. of phosphorus pentachloride in 30 cc. of dry chloroform. The mixture was allowed to stand for 1 hour at room temperature. The chloroform and phosphorus oxychloride were then removed under reduced pressure at 45°C. The residue was poured into water and shaken for 1 hour at room temperature to decompose any unchanged oxychloride. The oil was then extracted with ether, washed with dilute sodium hydroxide, then with water, and was finally dried over anhydrous sodium sulfate. When the ether was removed the residue weighed 7 gm. and showed an optical rotation of

$$[\alpha]_D^{20} = \frac{- 0.36° \times 100}{1 \times 16.878} = - 2.13°$$

CONCLUSIONS.

1. Isobutylmethyl carbinol and benzylphenyl carbinols have been resolved into their active components.

2. Ethylmethyl, isobutylmethyl, and benzylphenyl carbinols have been halogenated by means of reagents of different types. In each of these three alcohols halogenation was always accompanied with a change in the direction of rotation.

3. Ethylmethyl and isobutylmethyl carbinols are chlorinated by thionyl chloride only under definite conditions and their racemization under these conditions is not easily avoided.

ON THE OXIDATION OF SECONDARY MERCAPTANS INTO CORRESPONDING SULFONIC ACIDS.*

BY P. A. LEVENE AND L. A. MIKESKA.

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It was stated in previous publications[1] of this series that the aim of the work was to establish configurational relationships between amino, hydroxy, and halogen derivatives. In the fourth paper of this series, some tentative conclusions were drawn regarding the configurational relations between α-amino and α-hydroxy acids which were based on the observations made on the optical behavior of α-mercapto and α-sulfonic acids.

In the present communication, it will be shown that on the basis of observations on mercapto and sulfonic derivatives of secondary alcohols, certain deductions are permissible concerning the configurational relationships of secondary alcohols and corresponding halides.

In the first communication of this series the following relationships were shown to exist.

$$CH_3-\underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}}-C_6H_{13} \rightarrow CH_3-\underset{\underset{Cl}{|}}{\overset{\overset{H}{|}}{C}}-C_6H_{13} \rightarrow CH_3-\underset{\underset{SH}{|}}{\overset{\overset{H}{|}}{C}}-C_6H_{13} \rightarrow CH_3-\underset{\underset{OH \cdot SO_2}{|}}{\overset{\overset{H}{|}}{C}}-C_6H_{13}$$

| Dextro. | Levo with substitution. | Dextro. | Levo without substitution. |
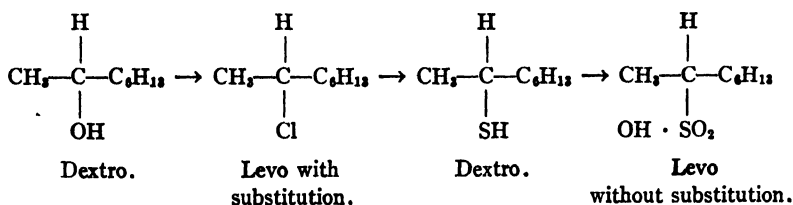
In a later communication it was shown that identical relationships existed in the analogous derivatives of ethylmethyl carbinol. In the present communication it will be shown that identical relationships exist between the analogous derivative of isobutylmethyl

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* This is the sixth paper of the series on Walden inversion.

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473; lx, 1, 685; 1925, lxiii, 85.

carbinol and of benzylphenyl carbinol. The oxidation of the mercapto into the sulfonic derivatives of all these alcohols is accompanied with a change of direction of rotation. Since the differences in the polarity between the OH group and Cl is qualitatively similar to those between the $-SH$ and $-SO_2OH$ groups, it may be concluded that the same configurational relationships exist between the alcohols and halides as between the mercapto and the sulfonic acid derivatives. Hence, it may be concluded that the series of alcohols thus far investigated by us are configurationally related to the halides which rotate in the opposite direction and are enantiomorphously related to those which rotate in the same direction. Attention will be directed next to three other types of alcohols; namely, to methylphenyl carbinol and its homologues, to isopropylphenyl carbinol and its homologues, and, finally, to those in which the aromatic radicles are attached directly to the asymmetric carbon atom.

The case of methylphenyl carbinol presents particular interest in view of the fact that the substitution by halogen of its hydroxyl is accompanied under some conditions with Walden inversion. A decision as to when the inversion occurs will be reached after the mercapto and the sulfonic derivatives of the alcohol have been prepared.

It may be mentioned here that 2-chloroisohexane, on conversion into the mercapto derivative, is more readily racemized than any of the other halides thus far employed by us.

2-Mercaptoisohexane.—28 gm. of *l*-2-iodoisohexane, with an optical activity of

$$[\alpha]_D^{20} = \frac{-1.24° \times 100}{1 \times 6.855} = -18.08°$$

were treated with two equivalents of alcoholic potassium hydrogen sulfide and heated under a return condenser at 60–65°C. for 5 hours. The mixture was then poured into water, whereupon an oil separated on the surface of the aqueous layer. This was separated, washed with a little water, and dried over sodium sulfate. The mercaptan obtained weighed 22 gm. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+0.48° \times 100}{1 \times 8.042} + 5.97°$$

That the above reaction was accompanied with considerable racemization is evident from the fact that in another experiment, carried out under similar conditions, the rotation of the original iodide was

$$[\alpha]_D^{20} = \frac{-1.13° \times 100}{1 \times 6.080} = -18.58°$$

from which a mercaptan was obtained with a rotation of

$$[\alpha]_D^{20} = \frac{+0.64° \times 100}{1 \times 2.921} = +21.21°$$

2-Isohexane Sulfonic Acid.—5 gm. of the mercaptan obtained in the experiment described above were oxidized by heating under a return condenser for 1½ hours with 8 cc. of concentrated nitric acid and 2 cc. of water. The substance was then transferred to an evaporating dish and concentrated on the steam bath almost to dryness. A little water was added and the mixture again evaporated almost to dryness. This was repeated until all the nitric acid was removed. The residue was then taken up with water and treated with an excess of barium carbonate. The solution was filtered hot. On concentration, the barium salt precipitated. It was recrystallized from a little water and analyzed.

0.0956 gm. substance: 0.0472 gm. BaSO₄ (for S).
0.1912 "   "   : 0.1850 "   "   (" Ba).
$C_{12}H_{26}O_6S_2Ba$.   Calculated.   S 13.70, Ba 29.37.
                         Found.   " 13.29, " 29.05.

To determine the rotation of the salt 1.0632 gm. were dissolved in water and the volume was made up to 10 cc. Rotation was determined in 1 dm. tube.

$$[\alpha]_D^{20} = \frac{-0.52° \times 100}{1 \times 10.632} = -4.89°. \qquad [M]_D^{20} = -11.68°.$$

1 cc. of hydrochloric acid was then added, making up the volume to 11 cc. For free acid

$$[\alpha]_D^{20} = \frac{-0.46° \times 100}{1 \times 6.885} = -6.68°. \qquad [M]_D = -11.15°.$$

d-Benzylphenyl Mercaptomethane.—14 gm. of *l*-benzylphenyl chloromethane

$$[\alpha]_D^{20} = \frac{-1.28° \times 100}{1 \times 11.998} = -10.66°$$

were treated with 3 mols of alcoholic potassium hydrogen sulfide solution. The mixture was allowed to stand at the room temperature for $1\frac{1}{2}$ hours which was followed by heating on the steam bath for 2 hours. In order to isolate the mercaptan, the reaction mixture was poured into water and the mercaptan extracted with ether. It was washed with water and dried over sodium sulfate. The ether was removed under reduced pressure at 50°C. At a higher temperature, the mercaptan slowly decomposed to stilbene and hydrogen sulfide. Its rotation was found to be

$$[\alpha]_D^{20} = \frac{+\ 0.58° \times 100}{1 \times 6.774} = +\ 8.56°$$

l-1, 2-Diphenylethyl Sulfonic Acid.—3 gm. of benzylphenyl mercaptomethane were dissolved in a mixture of acetone and water. Acetone solution of potassium permanganate was then added at room temperature until the permanganate was no longer being consumed. The excess of permanganate was then removed by adding a little more mercaptan. The manganese dioxide was then filtered off and the filtrate concentrated under reduced pressure. Two immiscible substances, consisting of an oil and the aqueous layer, were obtained. The oil was extracted with ether, washed with water, and dried over sodium sulfate. It showed positive rotation, but was not further investigated.

The aqueous layer was concentrated under reduced pressure. A semicrystalline salt was obtained. This was dried, analyzed, and its optical rotation determined.

For the salt $[\alpha]_D^{20} = \dfrac{-\ 2.06° \times 100}{1 \times 3.817} = -\ 53.96°.$ $[M]_D = -\ 159.5°.$

For the acid $[\alpha]_D^{20} = \dfrac{-\ 1.70° \times 100}{2 \times 2.335} = -\ 36.40°.$ $[M]_D = -\ 95.36°.$

0.1338 gm. substance: 0.1082 gm. BaSO$_4$ (for S).
0.0941 "   "   : 0.0259 " K$_2$SO$_4$ (" K).
          C$_{14}$H$_{13}$SO$_3$K. Calculated. S 10.67, K 13.02.
                    Found. . " 11.11, " 12.35.

THE DISSOCIATION CONSTANTS OF PLANT NUCLEO-TIDES AND NUCLEOSIDES AND THEIR RELATION TO NUCLEIC ACID STRUCTURE.

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I. INTRODUCTION AND THEORY.

Several theories have been advanced regarding the mode of union between the nucleotides in the nucleic acids. One possible mode of union is an "ether" linkage from one carbohydrate group to another. Another possible linkage is from the phosphoric acid group of one nucleotide to the carbohydrate group of the next, thus leading to a complex "ester."

The theory of the latter linkage has been supported in the case of animal (thymus) nucleic acid by direct chemical proof. This "ester" structure proposed by Levene has not been disputed and additional evidence in its favor will be presented in this paper.

The structure of plant (yeast) nucleic acid has, on the other hand, been subject to considerable controversy. According to Jones' original theory, the four nucleotides which compose this molecule are linked through the sugar groups. His later structure called for one such (*ether*) linkage and two *ester* linkages. According to Levene, however, all three linkages are *ester* linkages in both plant and animal nucleic acid.

As a possible means for determining the correct structure, we may observe that, in addition to the acidic and basic groups on the "bases,"[1] there should be *eight* ionizable hydrogen atoms on the phosphoric groups, according to Jones' original structure; *six*, according to his later structure; and *five*, according to the structure of Levene.

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[1] We retain the old (but misleading) term "base" to refer to a purine or pyrimidine derivative, even though some are acids or ampholytes.

Unfortunately, the determination of the total number of ionizable groups by direct titration (either colorimetric or electrometric) is prevented by the insolubility in acid solution, the possibility of hydrolysis, and the difficulty of working in strongly acid and, particularly, in strongly alkaline solutions. (As may be seen from the results in this paper, even the nucleotides give uncertain results in alkaline solutions.)

The problem can be solved, therefore, only by calculating the number of groups which should dissociate *within a certain pH range,* according to each of the proposed structures; and then determining the actual values experimentally. For this purpose, we have determined the dissociation constants of the nucleosides and the nucleotides, in order to be able to predict the constants for nucleic acid. These experimental values are presented in this paper.

II. RESULTS.

Table I gives the pK' value[2] of each ionizable group of each plant nucleoside and nucleotide as obtained by electrometric titration. Three of the four constants of guanylic acid have also been determined by Hammarsten.

Since a nucleoside consists of the (sugar–"base") complex, the only ionizable groups are those of the "base." In the nucleotides $(PO(OH)_2 \cdot O\text{–sugar–"base"})$ we have, in addition, two ionizable H atoms on the phosphoric group.

The consistency of each type of group is demonstrated in Fig. 1 in which the degree of dissociation is plotted against pH. The dotted curves refer to nucleosides and the solid lines to nucleotides. The curves for the phosphoric groups (lighter lines) show that these pK' values correspond approximately to (but are a little lower than) the first and second pK values of phosphoric acid (*i.e.,* 2.0 and 6.8).

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[2] The term pK' (which equals $-\log K'$) is used to refer to the constant not corrected for activity. Thus for an acid group: $K' = K \dfrac{f_i}{f_u}$ where f_u is the activity coefficient of a molecule in which the group in question is unionized, and f_i is the activity of a molecule when that group is ionized. All the constants given in this article are uncorrected. The corrected constants for acid groups would be about 0.1 pH unit higher; and for amino groups, about 0.1 pH unit lower.

The amino groups are also essentially alike and have constants in the same order of magnitude as aniline (pK = 4.7), except in the guanine derivatives. The constants of the first hydroxyl groups (except in the cytosine derivatives) are essentially the same as phenol (pK = 10). The difference between the two hydroxyl pK' values in

TABLE I.

pK' Values of Nucleosides and Nucleotides.

Nucleosides.	Nucleotides.	See Table No.	See Fig. No.	First H_2PO_4R ionization.	NH_2 group hydrolysis.	Second H_2PO_4R ionization.	First OH group.	Second OH group.
Adeno-sine.		II	1, A		3.45			
	Adenylic acid.	VIII	1, A	0.89	3.70	6.01		
Guano-sine.		III	1, B		1.6		9.16	
	Guanylic acid.	IX	1, B	0.7	2.3	5.92	9.36	
Cytidine.		IV	1, C		4.22		12.3	
	Cytidine phos-phoric acid.	X	1, C	0.80	4.24	5.97	(13.2)	
Uridine.		V	1, D				9.17	12.52
	Uridine phos-phoric acid.	XI	1, D	1.02		5.88	9.43	(13.9)

(Purine derivatives. — first two row-groups; Pyrimidine derivatives. — last two row-groups.)

In addition, the "base," *uracil*, was found to have pK' values of 9.28 and 13.56, due to its two hydroxyl groups.

Inosine (deaminated adenosine) has a value of 8.72 for its hydroxyl group (compare with the phenolic group of guanosine).

the uracil derivatives (*i.e.*, $pK_{a1}'-pK_{a2}'$) corresponds with the molecular dimensions and electrostatic forces.

The pK' values below 1, and particularly those above 12 or 13

are not accurate owing to experimental difficulties. The other values are reasonably accurate (but are not corrected for activity). There is every reason to believe that the animal nucleosides and nucleotides should have amost identical constants with those from plants.
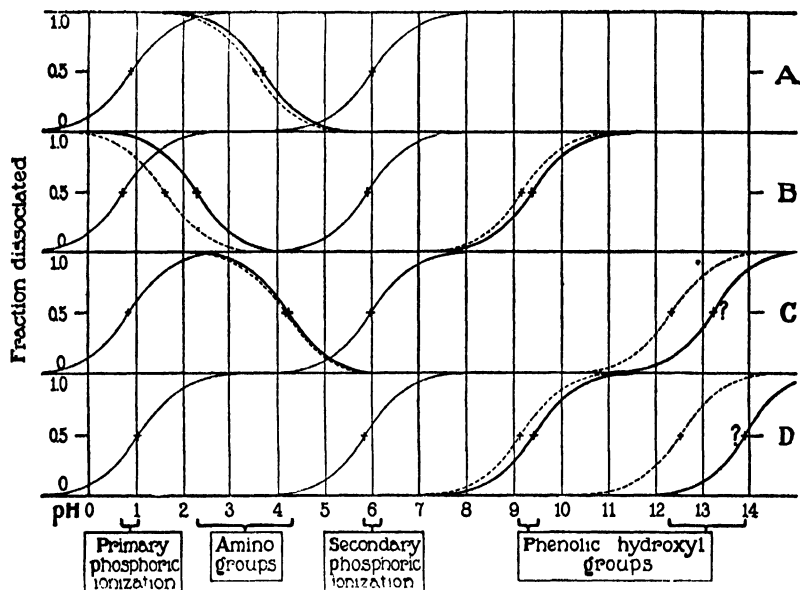
FIG. 1. Dissociation curves of the nucleotides (solid lines) and nucleosides (dotted lines). The lighter lines represent the dissociation of the phosphoric groups. The curves for the amino groups are reversed, thus representing their ionization.

A = adenylic acid and adenosine; B = guanylic acid and guanosine; C = cytidine phosphoric acid and cytidine; and D = uridine phosphoric acid and uridine.

III. BEARING ON THE STRUCTURE OF NUCLEIC ACID.

If we collect all the pK′ values found in the "bases" of the four nucleotides, we obtain the values represented in Fig. 2, A. These represent the pG′ values[3] of these groups which will be found in

[3] The G' constants are those found by treating the titration data as if the solutions contained equivalent quantities of monovalent acids. When the pG′ values are two or more pH units apart the G' constants equal the classical K' constants. For any two pG′ values which are closer together we have the relation:

nucleic acid according to either of the proposed structures; providing the groups are sufficiently distant in space to eliminate the mutual electrostatic effect. (The actual distances are large enough to make this effect small.)

In Fig. 2, B are the phosphoric pG' values to be expected from
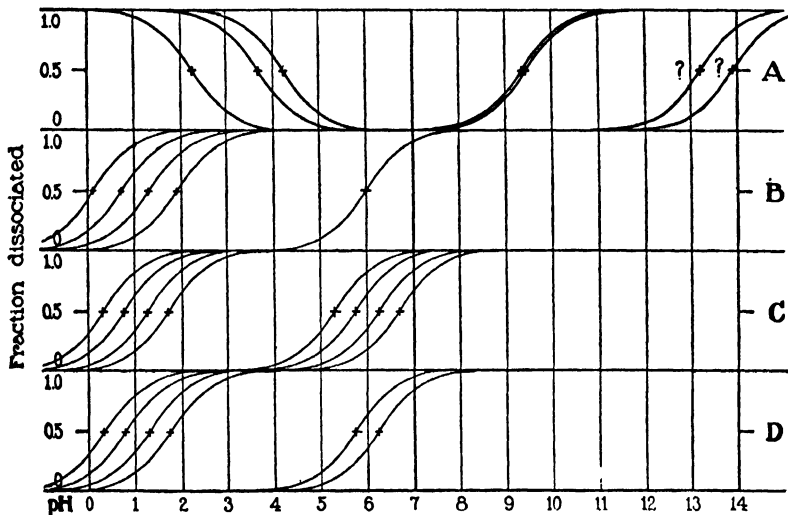
FIG. 2. Predicted approximate dissociation curves for nucleic acid.
A = phenolic and amino groups on the "bases" (according to any structure); B = phosphoric groups according to Levene's structure for plant (or animal) nucleic acid; C = phosphoric groups according to Jones' original structure for plant nucleic acid; and D = phosphoric groups according to Jones' later structure for plant nucleic acid.

Levene's theory of either plant or animal nucleic acid. They are spread apart to an extent roughly calculated for the distances between the groups (in space) and the electrostatic forces. Similarly, Fig. 2, C gives the dissociation curves of the phosphoric pG' values according

$$K_1' = G_1' + G_2' \qquad \text{and} \qquad (1)$$

$$\frac{1}{K_2'} = \frac{1}{G_1'} + \frac{1}{G_2'} \qquad\qquad (2)$$

When there are three or more groups close together, similar formulas may be used to calculate the K' constants. Since the G' constants are more useful for the present purpose, we need not consider the K' constants. For the nucleosides and nucleotides the K' constants may be considered equal to the corresponding G' constants, but this is not true for nucleic acid.

to Jones' original theory, and Fig. 2, *D* for his later theory of plant nucleic acid.

The essential difference between these structures lies in the number of "secondary" phosphoric groups (*i.e.*, the pG' values near pH 6). Any method for determining either the number of groups in that range or for determining the total number of groups below pH 8 (or any higher pH) will distinguish between the structures.

It will be seen from Fig. 2 that the following number of (acid) groups are ionized at the indicated pH values.

pH................................	5.0	8.0	11.0
Levene's structure........................	4	5	7
Jones' original structure.................. ..	4	8	10
" later "	4	6	8

Thomas and Dox[4] have analyzed sodium and sodium-ammonium salts of plant (yeast) nucleic acid and have found from 4 to 7 or 8 equivalents of alkali in solutions ranging from acid to alizarin, to alkaline to phenolphthalein. By disregarding the phenolic dissociation of the "bases," they assumed that this amount of alkali was combined with the phosphoric groups and that Jones' theory was supported. It will be seen from Fig. 2, however, that solutions which are alkaline to phenolphthalein (pH 10 or above) are in the range of dissociation of the phenolic groups, so that the data of Thomas and Dox support the theory of Levene rather than that of Jones. Their solutions containing only 5 or 6 equivalents of alkali were alkaline to phenolphthalein which agrees with Levene's theory[5] and not with Jones'. Only one of their seventeen solutions definitely disagrees with the predicted values for Levene's structure. That

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[4] Thomas, A., and Dox, A. W., *Z. physiol. Chem.*, 1925, cxlii, 1.

[5] Since they give no exact pH values it is hard to interpret the data of Thomas and Dox. A solution containing 5 equivalents of alkali should have a pH between 7 and 8 (according to Levene's structure) and a slight excess of alkali (*e.g.*, 0.2 equivalent) would bring it in the range where phenolphthalein would show pink and might be called "alkaline." (See Series III, Table II.) Certainly the data do not correspond with either structure of Jones, since these would give solutions distinctly acid to phenolphthalein with 5 (or with 5.2 or even 5.8) equivalents of alkali.

solution (Series I, Preparation *a*) was neutral to phenolphthalein but contained only 4 (instead of 5) equivalents of alkali (Jones' original structure would require 8, and his later structure, 6). The results of Thomas and Dox are summarized in Table II.

TABLE II.

Data of Thomas and Dox, on the Number of Equivalents of Base Combined with Nucleic Acid at Various Reactions.

Series	I			II		III		IV		V	
	Eq.*	Aliz.	Pp.	Eq.	Pp.	Eq.	Pp.	Eq.	Pp.	Eq.	Pp.
Preparation *a*.	4†	Alk.	Neut.†	7	Alk.	5	Alk.	7	Alk.	8	Alk.
" *b*.	4	"	Acid.	4	Acid.	5	"	7	"	8	"
" *c*.	4	"	"	4	"	5	"	6+	"	7	"
" *d*.	4	Acid.	"	4	"						

* Eq. = number of equivalents of base (sodium plus ammonia); Aliz. = reaction toward alizarin; Pp. = reaction toward phenolphthalein; Alk. = alkaline; Neut. = neutral.

† Series I, Preparation *a*, is the only one which does not correspond with Levene's structure.

We have made complete titration curves of nucleic acid and although our present values on yeast nucleic acid are unsatisfactory, we find the following tentative pG' values for thymus nucleic acid (calculated by a graphical modification of the "buffer value" method of Van Slyke[6]) between pH 3 and 10. The approximate predicted

	pG_1'	pG_2'	pG_3'	pG_4'	pG_{10}'	pG_{11}'	pG_{12}'	pG_{13}'
Predicted for Levene's structure (plant or animal)	3.7	4.2	(6.0)	9.4	9.5			
Found by Levene and Simms (animal)	4.0	4.3	6.3	9.5	9.7			
Found by Hammarsten[7] (animal)	3.7	4.3	5.2					
Predicted for Jones' original structure (plant)	3.7	4.2	(5.4)	(5.8)	(6.2)	(6.6)	9.4	9.5
Predicted for Jones' later structure (plant)	3.7	4.2	(5.8)	(6.2)	9.4	9.5		

The values in the range from pH 4.5 to 9 are given in bold faced type. It is evident that there is *but one pG* in this range; which agrees with the structure of Levene.

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[6] Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.

[7] Hammarsten, E., *Biochem. Z.*, 1924, cxliv, 383. The formulas used by

values (see Fig. 2) are given for comparison and also some pK' values from constants obtained by Hammarsten[7] on thymus nucleic acid. These values for thymus nucleic acid are only tentative and we also intend to obtain better data on yeast nucleic acid. We present the results in this paper on nucleosides and nucleotides as a preliminary communication.

IV. EXPERIMENTAL.

Sufficient material was weighed out in each case (allowing for moisture) to make a mother solution twice the strength indicated and sometimes containing exactly 1, 2, or 3 equivalents of molar strong acid or alkali. From these mother solutions, 5.0 cc. samples were diluted to 10.0 cc. (sometimes 2.5 cc. samples were diluted to 5.0 cc.) in volumetric flasks, after the required amount of tenth molar acid or alkali had been added.[8] These solutions had the indicated concentrations and, "equivalents of base" $\left(\dfrac{B-A}{C}\right)$. Their pH values were determined and are given in Tables III to XII.

The constants are calculated by the formulas:

$$G_s' = H \frac{\alpha_s}{1 - \alpha_s} \quad \text{and} \tag{3}$$

$$B' + y = \frac{h + B - A - oh}{C} + y = \alpha_1 + \alpha_f + \alpha_s + \text{etc.} = \Sigma\alpha \tag{4}$$

Hammarsten for calculating the constants are theoretically incorrect when applied to nucleic acid, and give values which are only roughly correct. We have not recalculated his data since the experimental error is large and also because it would be more important to know the values of the constants above pH 6 than to have more accurate values of those which he determined.

His formulas make correction for incomplete dissociation of groups with lower pG' values, but not for partial dissociation of groups with higher pG' values. Both corrections are equally necessary.

[8] It is interesting to observe that in neutralizing some alkaline solution of guanosine with HCl, a clear, transparent, viscous gel was first produced. In the course of a few seconds, small crystals formed which increased in number as the viscosity simultaneously dropped, until a thin watery suspension of finely precipitated material remained. The same cycle took place with another solution containing alcohol but was prolonged over a period of several minutes.

where G_z' is a constant (not corrected for activity) which, in all the nucleosides and nucleotides, is equal to either a dissociation constant (K_a') of an acid group or the hydrolysis constant (K_b') of a basic group. (There are many compounds, however, in which the G' constants cannot be considered equal to the K' constants, but the relation can then be found by equations (1) and (2) in Foot-note 3.)

$\dfrac{B' = h + B - A - oh}{C}$ is the "corrected equivalents of base."

y = number of groups which ionize as bases (amino groups).

h = hydrogen ion *concentration*, where H is its *activity*.

oh = hydroxyl " " " OH " " "

B = molar concentration of strong base (NaOH).

A = " " " " acid (HCl).

C = " " " substance.

Z = any subscript. (The G' constants are numbered in order of their numerical value.)

When the pG′ values are so close together that formulas (1) and (2) must be used, α_1, α_2, etc., do not correspond to any molecular or ionic concentrations. However, in cases such as the nucleosides and nucleotides where the pG′ values are far apart, α_z is the fraction of the substance in that form which predominates at a higher pH, and $(1 - \alpha_z)$ is the fraction in that form which predominates at a lower pH than pG_z', whether the group is acidic or basic.

All pH values were determined at 25.0°C. in a water-jacketed bubbling electrode. The pH of tenth normal hydrochloric acid was taken as 1.090 and the liquid junction potential with the saturated KCl bridge was assumed constant. The data are given in Tables III to XII.

TABLE III.
Adenosine. (0.0500 Molar.)

pH	$\frac{B-A}{C}$	B'	α	pG'
4.41	−0.100	−0.100	0.900	3.45
4.06	−0.200	−0.199	0.801	3.46
3.84	−0.300	−0.279	0.721	3.47
3.60	−0.400	−0.394	0.606	3.41
3.30	−0.600	−0.589	0.411	3.46
2.89	−0.800	−0.771	0.229	3.41
2.43	−1.000	−0.917	0.083	3.47
Amino group: pK$_b'$ =..				3.45

TABLE IV.
Guanosine. (0.0250 Molar.)

pH	$\frac{B-A}{C}$	B'	α_1	pG$_1'$	α_2	pG$_2'$
1.543	−1.800	−0.513	0.487	1.57		
1.14	−3.800	−0.755	0.245	(1.66)		
9.88	0.90	0.895			0.895	(8.95)
9.83	0.84	0.835			0.835	9.13
9.78	0.80	0.795			0.795	9.20
Amino group: pK$_b'$ =....................				1.6		
Hydroxyl group: pK$_a'$ =......................				....	....	9.16

TABLE V.
Cytidine (Sulfate). (0.0500 Molar.)

(B' is here calculated as if an equivalent of H_2SO_4 had been added to a solution of cytidine; *e.g.*, $\frac{A}{C} = 1$.)

pH	$\frac{B-A}{C}$	B'	α_1	pG$_1'$	α_2	pG$_2'$
3.33	−0.900	0.890	0.110	4.23		
3.84	−0.700	0.698	0.302	4.21		
4.22	−0.500	0.500	0.500	4.22		
4.58	−0.300	0.300	0.700	4.21		
11.83	0.500	0.300			0.300	12.20
12.05	0.650	0.320			0.320	12.38
12.27	0.900	0.35			0.35	12.54
12.20	1.000	0.54			0.54	12.27
Amino group: pK$_b'$ =....................				4.22		
Hydroxyl group: pK$_a'$ =......................				....	....	12.3

TABLE VI.

Uridine.   (0.0500 Molar.)

pH	$\frac{B-A}{C}$	B'	α_1	pG_1'	α_2	pG_2'
2.04	-0.200	0	0		0	
8.64	0.200	0.200	0.200	9.24		
8.73	0.300	0.300	0.300	9.10		
9.03	0.400	0.400	0.400	9.16		
9.16	0.500	0.500	0.500	9.17		
9.34	0.600	0.ο00	0.600	9.17		
9.74	0.800	0.798	0.798	9.17		
11.88	1.40	1.18			0.18	12.55
12.07	1.ο0	1.26			0.26	12.52
12.19	1.80	1.33			0.33	12.50
12.31	2.00	1.39			0.39	12.51
Hydroxyl groups $\begin{cases} pK_{a1}' = \ldots\ldots\ldots\ldots\ldots \\ pK_{a2}' = \ldots\ldots\ldots\ldots\ldots \end{cases}$				9.17		12.52

TABLE VII.

Uracil.   (0.0500 Molar.)

(The last three measurements were made in 0.500 molar solutions.)

pH	$\frac{B-A}{C}$	B'	α_1	pG_1'	α_2	pG_2'
8.66	0.200	0.200	0.200	9.26		
9.10	0.400	0.400	0.400	9.28		
9.46	0.600	0.600	0.600	9.28		
9.87	0.800	0.798	0.798	9.28		
12.13	1.400	1.025			0.025	(13.70)
12.39	1.800	1.065			0.065	13.55
12.49	2.000	1.080			0.080	13.55
13.11	1.600	1.23			0.23	13.62
13.22	1.800	1.31			0.31	13.57
13.32	2.000	1.38			0.38	13.53
Hydroxyl groups $\begin{cases} pK_{a1}' = \ldots\ldots\ldots\ldots\ldots \\ pK_{a2}' = \ldots\ldots\ldots\ldots\ldots \end{cases}$				9.28		13.56

TABLE VIII.

Inosine. (0.0240 Molar.)

pH	$\dfrac{B - A}{C} = B' = \alpha$	pG'
9.40	0.833	8.70
8.96	0.625	8.74
8.57	0.417	8.72
8.14	0.208	8.72
Hydroxyl group: $pK_a' = $..........................		8.72

TABLE IX.

Adenylic Acid. (0.0250 Molar.)

(pG$_2'$ was calculated before pG$_1'$ in order that it might be used to obtain the values of α_2 in the fourth column.)

pH	$\dfrac{B - A}{C}$	B'	α_2 (calculated).	$\alpha_1 = HB' - \alpha_2$	pG$_1'$	α_2	pG$_2'$	α_3	pG$_3'$
(2.08)	−0.400	−0.03	0.02	0.95	(0.80)				
(1.94)	−0.600	−0.08	0.02	0.90	(0.99)				
1.707	−1.000	−0.12	0.01	0.87	0.89				
1.223	−3.000	−0.31	0.01	0.68	0.89				
3.17	0.200	0.231				0.231	3.69		
3.55	0.400	0.413				0.413	3.70		
3.71	0.500	0.509				0.509	3.69		
3.90	0.600	0.606				0.606	3.71		
4.34	0.800	0.802				0.802	3.74		
5.49	1.200	1.200						0.200	6.06
5.84	1.400	1.400						0.400	6.01
6.00	1.500	1.500						0.500	6.00
6.17	1.600	1.600						0.600	6.00
6.62	1.800	1.800						0.800	6.02
9.34	2.000	2.000							
11.53	2.200	2.000							
First phosphoric ionization: $pK_{p1}' = $....				· 0.89					
Amino group: $pK_b' = $....				....	....		3.70		
Second phosphoric ionization: $pK_{p2}' = $....				....	....	....		....	6.01

TABLE X.

Guanylic Acid. *(0.0250 Molar.)*

(pG$_2'$ was calculated before pG$_1'$ in order that it might be used to obtain the value of α_2 in the fourth column.)

pH	$\dfrac{B-A}{C}$	B	α_2 (calculated).	$\alpha_1 = HB' - \alpha_2$	pG$_1'$	α_2	pG$_2'$	α_3	pG$_3'$	α_4	pG$_4'$
1.10	−4.00	−0.46	0.06	0.48	(1.13)						
1.21	−3.00	−0.24	0.07	0.69	0.86						
1.39	−2.00	−0.17	0.10	0.73	0.97						
1.61	−1.000	+0.102	0.160	0.940	0.41						
1.78	−0.600	0.145	0.218	0.927	0.67	0.222	2.32				
1.81	−0.500	0.195	0.231	0.964	0.39						
2.08	0	0.373				0.411	2.24				
2.66	0.600	0.695				0.695	2.30				
3.07	0.800	0.838				0.838	2.36				
4.37	1.000	1.002									
5.34	1.200	1.200						0.200	5.94		
5.77	1.400	1.400						0.400	5.94		
6.09	1.600	1.600						0.600	5.92		
6.49	1.800	1.800						0.800	5.89		
7.44	2.000	2.000									
8.79	2.200	2.200								0.200	9.39
9.24	2.400	2.400								0.400	9.38
9.53	2.600	2.598								0.598	9.36

First phosphoric ionization:	pK$_{p1}'$ =.....	0.7				
Amino group:	pK$_b'$ =.....	......		2.3		
Second phosphoric ionization:	pK$_{p2}'$ =.....	......	......	......	5.92	
Hydroxyl group:	pK$_a'$ =.....	......	......	......	......	9.38

"First," "second," and "third" constants of Hammarsten..............................	2.4	6.1	9.7

TABLE XI.

Cytidine Phosphoric Acid.   *(0.0259 Molar.)*

pH	$\frac{B-A}{C}$	B'	α_1	pG_1'	α_2 (calculated).	$\alpha_2 = B' - \alpha_2$	pG_2'	α_3 (calculated).	$\alpha_3 = B' - \alpha_2$	pG_3'	α_4	pG_4'
1.217	−3.000	−0.28	0.72	0.81								
1.397	−2.000	−0.26	0.80	0.80								
(2.30)	−0.20	0.02										
(2.80)	0	0.07										
2.58	+0.200	0.212			0.01	0.20	(4.15)					
4.04	0.400	0.404			0.012	0.392	4.23					
4.21	0.500	0.503			0.017	0.486	4.23					
4.38	0.600	0.602			0.025	0.577	4.25					
5.19	1.000	1.000			0.13	0.87	(4.31)					
5.51	1.200	1.200						0.952	0.248	5.99		
5.82	1.400	1.400						0.977	0.423	5.95		
6.15	1.600	1.600						0.988	0.612	5.95		
6.58	1.800	1.800						1.000	0.800	5.98		
7.77	2.000	2.061										
11.93	2.574	2.050									0.050	13.21
12.18	2.970	2.08									0.08	13.24
12.49	3.960	2.13									0.13	13.31

First phosphoric ionization: $pK_{p1}' = \ldots.$ 0.80

Amino group: $pK_b' = \ldots$ $\ldots\ldots\ldots\ldots$ 4.24

Second phosphoric ionization: $pK_{p2}' = \ldots$ $\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots$ 5.97

Hydroxyl group: $pK_a' = \ldots.$ $\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots$ 13.2

TABLE XII.

Uridine Phosphoric Acid. *(0.0250 Molar.)*

(The ammonium salt was used for determining pG_1 and pG_2, but a sample of free acid was used at higher pH values.)

pH	$\dfrac{B-A}{C}$	B'	α_1	pG_1'	α_2	pG_2'	α_3	pG_3'	α_4	pG_4'
1.405	−1.000	0.76	0.76	(0.91)						
1.482	−0.800	0.68	0.68	(1.16)						
1.523	−0.600	0.74	0.74	1.16						
1.572	−0.400	0.802	0.802	0.97						
1.642	0.200	0.823	0.823	0.98						
1.726	0	0.843	0.834	1.00						
1.954	0.400	0.900	0.900	1.01						
2.145	0.400	0.922	0.922	1.07						
3.83	1.000	1.006								
5.27	1.200	1.200			0.200	5.87				
5.71	1.400	1.400			0.400	5.89				
6.07	1.600	1.600			0.600	5.90				
6.45	1.800	1.800			0.800	5.85				
9.03	2.280	2.280					0.280	9.44		
9.40	2.480	2.480					0.480	9.43		
9.77	2.680	2.676					0.676	9.44		
10.25	2.880	2.870					0.870	9.42		
11.16	3.08	2.99								
11.91	3.48	3.00								
12.17	3.88	3.00								
12.34	4.28	2.99								
12.83	7.08	3.08							0.08	13.9

(Other readings unsatisfactory.)

First phorphoric ionization:	pK_{p1}' =.......			1.02						
Second phosphoric ionization:	pK_{p2}' =.......			......	......	5.88				
First hydroxyl group:	pK_{a1}' =......			......	......	......	......	9.43		
Second hydroxyl group:	pK_{a2}' =......			......	......	......	......	......	......	(13.9)

V. SUMMARY.

The fifteen dissociation constants of the four nucleotides which compose a plant nucleic acid molecule; and also the seven constants of the four corresponding nucleosides have been determined.

The amino groups give values near that of aniline; the hydroxyl groups give values in the order of magnitude of phenol; while the primary and secondary ionizations of the phosphoric groups are close to those of phosphoric acid.

The various structures proposed for nucleic acids should have essentially identical constants except in the range near pH 6.   In that range, Levene's structure for plant nucleic acid (and also his structure for animal nucleic acid) requires *one* group; while the structures proposed by Jones for plant nucleic acid call for *two* or *four* groups in the same range.

Data obtained by Thomas and Dox, although interpreted differently by them, support the structure of Levene for plant nucleic acid.

Tentative constants of animal (thymus) nucleic acid obtained by Levene and Simms also substantiate the structure of Levene for this substance.

THE NUMERICAL VALUES OF THE OPTICAL ROTATION OF METHYLATED GLUCONIC ACIDS AND OF THEIR SALTS.

By P. A. LEVENE and G. M. MEYER.

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The observations on the optical rotations of sugar acids and their salts and on the α substituted acids and their salts have led to the general conclusion that the rotations of the free acids have a direction to the left from their metallic salts for all those α-hydroxy acids in which the allocation of the hydroxyl is the same as that of carbon atom (2) of gulonic acid; that is, the difference between the numerical values of the rotations of the free acids and of their salts has a minus sign. The same is true for 2-aminohexonic acids and for α-amino acids. The rule is reversed for the acids in which carbon atom (2) has the configuration of mannonic acid. This rule held for all free acids thus far observed, and it was therefore suggested as a basis for differentiation between the members of the l and of the d series.

Observations on other derivatives of α-hydroxy acids are not quite so numerous. They were entirely lacking on the methyl ethers of sugar acids, and yet a knowledge of the optical behavior of these derivatives seemed desirable for the sake of finding an explanation for the differences in the rotations of free acids and of their salts.

From the observations of previous workers it is known that α-methoxy-l-propionic acid (derived from l-lactic acid (dextro)) has a molecular rotation for the free acid $[M]_D = -66°$ ($c = 3$ to 4); the Na salt has $[M]_D = -62.8°$; and other salts also show a decreasing levorotation whereas l-lactic acid is dextrorotatory and its salts are levorotatory.

In the case of malic acid, the d acid has a specific rotation ($c = 10$) of $+2.17°$ and the potassium salt $+6.78°$; on the other hand, d-methoxysuccinic acid has a specific rotation of $+33.3°$ ($c = 10.8$)

whereas the neutral potassium salt has the specific rotation of $+9.54°$ ($c = 12.16$).

For the sugar acids, to our knowledge, observations are recorded only on methylated mannonic acids; namely, on 2,3,5,6-tetramethyl and 2,3,4,6-tetramethyl mannonic acids.

Two peculiarities were observed on these two derivatives. The 2,3,5,6-tetramethyl mannonic acid was levorotatory whereas the 2,3,4,6-tetramethyl derivative was dextrorotatory. This difference in direction of rotation of two derivatives differing only in that one of the methyl groups in one was in position 4 and in the other, in position 5, seemed rather surprising. Furthermore, the difference between the rotations of the free acids and their sodium salts had a minus sign, as it is in the d-gluconic acid series of unsubstituted acids.

With a view of finding an explanation for the differences in the optical behavior of two substances so closely related, it was considered expedient to prepare a series of partially methylated and of completely methylated gluconic acids.

There was still another interest attached to the present investigation. It was pointed out some time ago by one of us that 2,5-anhydrogluconic acid and 2,5-anhydromannonic acid show exactly the same molecular rotations either as salts or as free acids. This peculiarity was explained by the rigidity of the structures of these two acids. However, *a priori*, another factor might have been held responsible for this phenomenon; namely, the absence of replaceable hydrogens on carbon atoms (2) and (5). The behavior of the methylated gluconic acids should furnish the data required for choosing between the two possible explanations. This problem may be dismissed without much discussion, since in all methylated gluconic acids, the molecular rotations of the acids differed from those of their salts. Thus, these observations lent support to the first of the two above mentioned theories.

The details of the optical behavior of the methylated gluconic acids are given in Table I.

From this table it is seen that in the series of methylated gluconic acids the difference in rotations of the free acids and their salt has a minus sign as is the case in the free gluconic acid. In this respect the methylated gluconic acids differ from mannonic acid.

The table further shows: first, that all methylated acids have a higher dextrorotation than the mother substance; second that two acids containing an equal number of methyl groups may have different molecular rotations when the methyl groups are differently distributed. Thus, 2,3,5- and 3,5,6-trimethyl gluconic acids have different molecular rotations. Particularly striking is the fact that

TABLE I.

Specific and Molecular Rotation of Methylated GluconicAcids.

Methylated derivative.	Solution in 1 equivalent NaOH.		The same solution neutralized.	
	$[\alpha]_D$	$[M]_D$	$[\alpha]_D$	$[M]_D$
2,3-Dimethyl..........................	+43.7	+97.46	+22.5	+50.17
3,5,6-Trimethyl.......................	+24.0	+56.88	−6.35	−15.05
2,3,5-Trimethyl.......................	+64.4	+152.62	+19.3	+55.74
2,3,5,6-Tetramethyl.........	+76.4	+191.76	+43.4	+108.93
2,3,4,5,6-Pentamethyl................	+53.7	+141.30	+22.5	+59.62

TABLE II.

Change in the Numerical Value* of Rotation Due to Substitution in Different Positions.

Carbon atom No.	$[M]_D$
2	+134.88
3	−63.02
4	−50.46
5	+55.16
6	+39.14

* These values are calculated from the molecular rotation in alkaline solution of Table I less that of gluconic acid $[M]_D = 25.60$. It is not implied that these values will hold for other combinations.

the pentamethylated acid has a lower molecular rotation than some of the partially methylated acids. From these the conclusion immediately follows that whereas the methylation of some of the hydroxyls augments the rotation of the mother substance, the methylation of the others caused a drop in the rotation.

In fact, from the data given in Table I, it is possible to calculate the effect on the total molecular rotation of the methylation of each

one of the carbon atoms separately. These values are given in Table II. Plus sign indicates an enhancing influence, minus sign a depressing one.

It is expected to obtain data on the molecular rotation of the methylated derivatives of the other sugar acids in the hope that a more comprehensive explanation of the optical behavior of these substances may be arrived at.

CONCLUSIONS.

1. In all methylated gluconic acids, the difference in the optical rotations of the free acids and of their salts has a minus sign.

2. The influence on the value of the molecular rotation of the methylation varies with the change in position of the methyl groups.

3. Further evidence is furnished to the theory that the peculiarity in the behavior of 2,5-anhydrosugar acids is due to the rigidity of their structures.

EXPERIMENTAL.

2,3-Dimethyl Gluconic Lactone.

Benzylidene Methyl Glucoside.—The method of Irvine[1] was slightly modified: 50 gm. of methyl glucoside, 300 cc. of freshly distilled benzaldehyde, and 50 gm. of anhydrous sodium sulfate contained in a distillation flask were heated in an oil bath at 145°C. for 2 hours and then at 165°C. for 5 hours: The benzaldehyde was removed as far as possible by distillation under reduced pressure (oil bath temperature 125°C.). The contents of the flask were extracted with dry neutral ethyl acetate and filtered from the sodium sulfate. On cooling with ice and salt the benzylidene compound was obtained as a crystalline mass. It was filtered on suction and washed with cold ether. This removed most of the coloring water and left the benzylidene glucoside as a nearly white crystalline powder. The ethyl acetate filtrate was concentrated under reduced pressure and poured into a large volume of petrolic ether. The precipitate was recrystallized from ethyl acetate The total yield of crude material from 100 gm. of glucoside was 145 gm. This product was recrystal-

[1] Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 580.

lized from methyl alcohol and it then had a melting point of 160–161° and analyzed as follows:

0.0966 gm. substance: 0.2138 gm. CO_2 and 0.0582 gm. H_2O.
$C_{14}H_{18}O_6$. Calculated. C 59.57, H 6.38.
              Found.   " 60.38, " 6.74.

2,3-Dimethyl Benzylidene Methyl Glucoside.—This was prepared from the benzylidene glucoside according to the directions of Irvine. It melted at 122–123°C. and had the following composition.

0.1028 gm. substance: 0.2322 gm. CO_2 and 0.0662 gm. H_2O.
$C_8H_{22}O_6$. Calculated. C 61.89, H 7.12.
              Found.   " 61.57, " 7.20.

2,3-Dimethyl Glucose.—The previous compound was boiled with 10 per cent hydrochloric acid for 30 minutes. The solution was cooled and the benzaldehyde removed in a separatory funnel with ether. The aqueous part was neutralized with barium carbonate and the methylated sugar isolated by the usual procedures. No attempt was made further to purify the product.

2,3-Dimethyl Gluconic Lactone.—35 gm. of the syrup obtained by the hydrolysis of dimethyl benzylidene glucoside were oxidized with bromine at 30–35°C. and constant stirring until it failed to reduce Fehling's solution. The excess bromine was removed under reduced pressure and the hydrobromic acid with silver carbonate according to the usual procedure.

The syrup which remained after removal of the solvent by distillation under diminished pressure was taken up in ether, the solution was dried with sodium sulfate, and the ether was removed by distillation under diminished pressure. The product was dried at 100°C. in a high vacuum (p = 0.05 mm.) and analyzed as follows:

0.1082 gm. substance: 0.1824 gm. CO_2 and 0.0682 gm. H_2O.
0.1760 "   "   : 0.4134 " AgI (Zeisel).
$C_8H_{14}O_6$ (mol. wt. 206). Calculated. C 46.70, H 6.80, CH_3 30.10.
                  Found.   " 45.97, " 7.05, " 31.0.

The product titrated as a lactone.

0.0863 gm. of substance was neutralized by 4.25 cc. of 0.1 N alkali,

equivalent to a molecular weight of 203. It had the following optical rotation in aqueous alcohol.

$$[\alpha]_D^{20} = \frac{+1.64° \times 100}{1 \times 2.81} = +58.5°$$

Sodium Salt.—0.4100 gm. substance was dissolved in 2.5 cc. N NaOH. This was heated at 80–90°C. for several hours and made up to 5 cc. with water.

$$[\alpha]_D^{20} = \frac{+3.62° \times 100}{1 \times 8.2} = +44.1°$$

Free Acid.—4 cc. of the solution used for the previous rotation were pipetted into a 10 cc. flask, cooled with ice and alcohol, and neutralized with 2.25 cc. N HCl.

After 24 hrs.

$$[\alpha]_D^{•} = \frac{+1.47° \times 100}{2 \times 3.28} = +22.5° \qquad [\alpha]_D^{20} = \frac{+2.65° \times 100}{2 \times 3.28} = +40.4°$$

The above procedure for determining the optical rotation of the salt and free acid was followed throughout.

3,5,6-Trimethyl Gluconic Lactone.

3,5,6-Trimethyl Monoacetone Glucose.—Monoacetone glucose in portions of 20 gm. was methylated with 125 gm. of methyl sulfate and 250 cc. of 30 per cent sodium hydroxide. The methylated sugar separated as an oil on the surface of the liquid. It was extracted with ether, dried with sodium sulfate, and the ether removed under reduced pressure. The syrup, 21 gm., was distilled. It boiled at 110°C., $p = 0.3$ mm., $n_D^{23} = 1.44914$.

It analyzed for the methoxyl as follows:

0.1098 gm. substance: 0.2842 gm. AgI (Zeisel).
$\qquad$ $C_{12}H_{22}O_6$. Calculated. OCH_3 35.4.
$\qquad$ Found. " 34.1.

3,5,6-Trimethyl Glucose.—The acetone sugar was hydrolyzed with 0.5 per cent hydrochloric acid and the trimethyl glucose which was isolated by the usual procedure was analyzed without previous distillation.

0.1102 gm. substance: 0.1992 gm. CO_2 and 0.0848 gm. H_2O.

$$C_9H_{18}O_6. \quad \text{Calculated.} \quad \text{C } 48.65, \text{ H } 8.11.$$
$$\text{Found.} \quad \text{ " } 49.22, \text{ " } 8.60.$$

3,5,6-Trimethyl Gluconic Lactone.—The trimethyl glucose was oxidized by bromine as already described. The hydrobromic acid was neutralized with the calculated amount of 1.0 N NaOH after a quantitative determination of the HBr by Volhard's method. The lactone was extracted with ether after the solvents had been removed under reduced pressure, and the ether solution treated as usual. The lactone which was obtained as a syrup was distilled and boiled at 155°, $p = 1$ mm. It analyzed as follows:

0.1186 gm. substance: 0.2114 gm. CO_2 and 0.0796 gm. H_2O.
0.1462 "   "   : 0.4806 " AgI (Zeisel).

$$C_9H_{16}O_6 \text{ (mol. wt. 220).} \quad \text{Calculated.} \quad \text{C } 49.05, \text{ H } 7.3, \text{ OCH}_3 \text{ 42.24.}$$
$$\text{Found.} \quad \text{ " } 48.60, \text{ " } 7.51, \text{ " } 43.39.$$

The product titrated as a lactone.

0.0802 gm. of substance was neutralized by 3.55 cc. of 0.1 N alkali, equivalent to a molecular weight of 225.

It had the following optical rotation in aqueous alcohol.

After 24 hrs.

$$[\alpha]_D^{20} = \frac{+1.25° \times 100}{1 \times 2.836} = +44.1° \qquad [\alpha]_D^{20} = \frac{+1.13° \times 100}{1 \times 2.836} = +39.8°$$

Sodium salt.

$$[\alpha]_D^{20} = \frac{+1.10° \times 100}{1 \times 4.57} = +24.0°$$

Free acid.

After 24 hrs.

$$[\alpha]_D^{0} = \frac{-0.23° \times 100}{2 \times 1.88} = -6.3° \qquad [\alpha]_D^{20} = \frac{+0.20° \times 100}{2 \times 1.828} = +5.4°$$

2,3,5-Trimethyl Gluconic Lactone.—2,3,5-trimethyl glucoside which was obtained by fractional distillation of methylated glucosides and which analyzed for methoxy 52.3 per cent, was converted by the usual procedure into the free sugar. The trimethyl glucose was oxidized with bromine by the method already described. The lactone distilled at 142°C., $p = 0.14$ mm., and analyzed as follows:

0.1066 gm. substance: 0.1904 gm. CO_2 and 0.0716 gm. H_2O.

0.1172 "   "   : 0.3726 " AgI (Zeisel).

$C_9H_{16}O_6$ (mol. wt. 220).   Calculated.   C 49.05, H 7.27, OCH_3 42.24.

                    Found.   " 48.70, " 7.51,   "   41.96.

The product titrated as a lactone.   0.0934 gm. substance was neutralized by 4.20 cc. 0.1 N alkali, equivalent to a molecular weight of 222.

It had the following optical rotation in aqueous alcohol.

After 24 hrs.

$$[\alpha]_D = \frac{+2.23° \times 100}{1 \times 2.452} = +90.8° \qquad [\alpha]_D^{20} = \frac{+1.35° \times 100}{1 \times 2.452} = +55.0°$$

Sodium salt.

$$[\alpha]_D^{20} = \frac{+1.57° \times 100}{1 \times 2.440} = +64.4°$$

Free acid.

After 24 hrs.

$$[\alpha]_D^0 = \frac{+0.75° \times 100}{2 \times 1.952} = +19.3° \qquad [\alpha]_D^{20} = \frac{+0.92° \times 100}{2 \times 1.952} = +23.5°$$

Tetramethyl Gluconic Lactone.—Tetramethyl glucose was oxidized with Br by the methods already described.   The lactone distilled at 128°C., $p = 0.8$ mm., and analyzed as follows:

0.1056 gm. substance: 0.1986 gm. CO_2 and 0.0730 gm. H_2O..

0.1069 "   "   : 0.4300 " AgI (Zeisel).   -

$C_{10}H_{18}O_6$.   Calculated.   C 51.20, H 7.7, OCH_3 53.00.

            Found.   . " 51.18, " 7.73,   "   53.44.

The lactone had the following optical rotation in aqueous alcohol.

After 24 hrs.

$$[\alpha]_D^{20} = \frac{+2.95° \times 100}{1 \times 2.78} = +106.1° \qquad [\alpha]_D^{20} = \frac{+1.45° \times 100}{1 \times 2.78} = +52.2°$$

Salt.

$$[\alpha]_D^{20} = \frac{+3.36° \times 100}{1 \times 4.4} = +76.4°$$

Acid.

After 24 hrs.

$$[\alpha]_D^0 = \frac{+1.5° \times 100}{2 \times 1.76} = +43.0° \qquad [\alpha]_D^{20} = \frac{+1.74° \times 100}{2 \times 1.76} = +49.5°$$

Pentamethyl Gluconic Acid.

Calcium gluconate in portions of 20 gm. was methylated with 125 cc. of dimethyl sulfate and 250 cc. of 30 per cent sodium hydroxide. The product was made neutral to Congo red with sulfuric acid and concentrated under diminished pressure to about 150 cc., filtering occasionally from the deposited sodium sulfate. It was then acidified with sulfuric acid and extracted with ether on a continuous extractor. After extracting in this manner for several days the ether solution was dried with anhydrous sodium sulfate and the ether removed under diminished pressure.

In this manner approximately 50 per cent by weight of the calcium gluconate employed was recovered as a syrup. The syrup contained only partially methylated gluconic acid having a methoxy value of 38 to 40 per cent. It was nearly completely soluble in methyl iodide and could therefore be further methylated by the method of Irvine without recourse to any extraneous solvent. The methyl iodide-silver methylation was twice repeated, using 140 gm. of methyl iodide and 100 gm. of silver oxide for each methylation. The methylated syrup, which was recovered with but slight loss, then contained approximately 62.0 per cent of OCH_3.

Further methylation by this method failed to increase the methoxy content. Likewise, after treatment with diazomethane, the product still analyzed for 62.6 per cent of OCH_3. This syrup distilled fairly constant at 112°C., $p = 0.5$ mm., and could not be fractionated.

15 gm. of this product were therefore hydrolyzed with an excess (105 cc.) of 1.0 N sodium hydroxide at 80–90° for 2 hours.

The above solution then had $[\alpha] = +5.80°$ and further heating for 1 hour did not change this value. The solution was therefore concentrated to 50 cc. under diminished pressure without undue application of heat and the product methylated with 80 cc. of dimethyl sulfate and 180 cc. of 30 per cent sodium hydroxide. The methylated product was recovered from this solution exactly as described above.

The syrup was fractionated and distilled mainly at 155°C., $p = 1$ mm.

It analyzed as follows:

0.1346 gm. substance: 0.2428 gm. CO_2 and 0.0980 gm. H_2O.

0.0998 " " : (Zeisel) 0.4308 gm. AgI.

$C_{11}H_{22}O_7$ (mol. wt. 266).   Calculated.   C 49.60, H 8.32, OCH_3 58.2.

                                      Found.   " 49.11, " 8.14, "   56.81.

The product titrated as an acid, 0.0882 gm. of substance being neutralized by 3.30 cc. of 0.1 N alkali equivalent to a molecular weight of 267.

Pentamethyl gluconic acid had the following optical rotation in water.

$$[\alpha]_D^{20} = \frac{+\,1.00° \times 100}{1 \times 4.44} = +\,22.5°$$

Sodium salt.

$$[\alpha]_D^{20} = \frac{+\,2.52° \times 100}{1 \times 4.7} = +\,53.7°$$

After neutralization after sodium salt.

$$[\alpha]_D^{20} = \frac{+\,0.85° \times 100}{2 \times 1.88} = +\,22.5°$$

BROMOLECITHINS.

I. FRACTIONATION OF BROMINATED SOY BEAN LECITHINS.*

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Lecithins prepared from animal organs or from plants contain two saturated and several unsaturated fatty acids. It has been assumed that this fact indicated the existence of several lecithins each containing only two fatty acids. The alternative view would be the one postulating the structure of lecithins as polylecithides, containing in one molecule all the fatty acids isolated from lecithins.

A priori, the simpler structure seems the more probable one, but up to the present, there has been no experimental evidence advanced which would give certain preference to one ot these theories over the other.

Attempts to fractionate the lecithins had been made by us before, but with little success. Recently it was decided to try to fractionate, not the free lecithins, but their bromo derivatives.

Several reasons suggested this method. First, our experience in separating cephalin from lecithin has shown that the task was more easily accomplished after the phosphatides had been hydrogenated. Second, it is known that the solubilities of the higher aliphatic bromo acids decrease with the increase in the number of bromine atoms. Thus hexabromostearic acid is much less soluble than tetrabromostearic acid.

Preparation of bromolecithins has been mentioned in patent literature, but the substances have not been utilized for theoretical purposes. The present investigation has been limited to lecithins extracted from soy beans.

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When a solution of such lecithins in petrolic ether is treated with a solution of bromine in the same solvent, a product insoluble in this solvent is formed.

Before attempting fractionation of the bromolecithins, the fatty acids of the whole material were separated and analyzed. From the biomine content of the material it seemed probable that it contained in the main two brominated acids besides the saturated. In accordance with this assumption, it has been possible to isolate from this material and to identify hexabromostearic and tetrabromostearic acids.

This find encouraged the attempt to fractionate the bromolecithins. Indeed we have succeeded in separating the hexabromolecithin in pure form, and a tetrabromo derivative in, a state sufficiently pure to warrant the assumption of the existence of an individual tetrabromolecithin. The dibromolecithin should be obtainable from the mother liquors of the insoluble bromolecithins.

Thus, the work on bromolecithins lends experimental support to the assumption that lecithins as isolated from animal and plant tissues are mixtures of individual lecithins each containing two fatty acids.

The nature of the saturated acids associated with each unsaturated acid will be the subject of the next investigation. The work will also be extended to lecithins prepared from animal tissues.

<div align="center">EXPERIMENTAL.</div>

The mixture of lecithins used as intermediary material was isolated as a cadmium chloride salt from the lipoid fraction of the soy bean in the manner described in a previous publication.[1] The lipoid mixture, freed from cadmium chloride with ammonia, analyzed as follows:

0.1069 gm. substance: 0.2501 gm. CO_2, 0.1007 gm. H_2O, and 0.0084 gm. ash.
0.1945 "   "   required (Kjeldahl) 2.63 cc. 0.1 N acid.
0.2931 "   "   : (fusion) 0.0410 gm. $Mg_2P_2O_7$.

[1] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1924-25, lxii, 759.

2 gm. substance were hydrolyzed with 10 per cent HCl.
          5 cc. required (Kjeldahl) 0.29 cc. 0.1 N acid.
          2 "   "   (Van Slyke) 0.09 cc. N_2 at 22°C., 748 mm.
      $C_{43}H_{86}O_9NP$. Calculated. C 65.26, H 10.95, N 1.77, P 3.92.
          Found.   " 63.80, " 10.54, " 1.89, " 3.88.

$$\frac{\text{Amino } N_2}{\text{Total } N_2} = \frac{3}{100}$$

Bromination of this material was accomplished most satisfactorily by dissolving the lipoid in 10 parts of gasoline (boiling at 40–50°C.) and adding the bromine (dissolved in 5 parts of gasoline) at such a rate that the temperature of the reaction mixture could be kept below $-5°$. As the reaction proceeded, the brominated lipoids gradually precipitated, giving rise to a characteristic color change in the solution. When the bromine was no longer decolorized the supernatant liquor was decanted, and the precipitated material was very thoroughly washed with fresh gasoline to remove any excess bromine. The yield of this crude material was theoretical. Finally the precipitate, which resembled lecithin in texture but was of a more silvery appearance, was dissolved in ether. From the ethereal solution a material was separated on standing at 0°C. which at this temperature seemed to consist of a light yellow, finely divided amorphous powder. On warming to room temperature, however, it coalesced to a typical lecithin-like mass, rather harder than the ordinary unsaturated lipoid but of the same light reddish brown color. This fraction analyzed as follows:

No. 187. 0.200 gm. substance required (Kjeldahl) 1.95 cc. 0.1 N acid.
          0.300 "   "   : (fusion) 0.0300 gm. $Mg_2P_2O_7$.
          0.1342 "   "   : (Carius) 0.1128 gm. AgBr.
              Hexabromostearyl stearyl lecithin, $C_{44}H_{84}O_9NPBr_6$.
              Calculated. N 1.09, P 2.42, Br 37.45.
              Found.   " 1.36, " 2.72, " 35.77.

The ether-soluble fraction of the brominated lipoids was precipitated by the addition of several volumes of gasoline and formed a shiny soft buttery mass, which was soluble in acetone, alcohol, and glacial acetic acid. It analyzed as follows:

No. 189. 0.2000 gm. substance required(Kjeldahl) 2.10 cc. 0.1 N acid.
          0.3000 "   "   : (fusion) 0.0310 gm. $Mg_2P_2O_7$.
          0.1296 "   "   : (Carius) 0.0988 " AgBr.

Tetrabromostearyl stearyl lecithin, $C_{44}H_{86}O_9NPBr_4$.
Calculàted. N 1.24, P 2.76, Br 28.50.
Found. " 1.47, " 2.88, " 32.44.

To effect further fractionation, this material was again dissolved in ether, and again a small amount of precipitate could be separated which was insoluble in ether at 0°. The more soluble portion was fractionated by the gradual addition of gasoline. The fraction which remained soluble in a 50 per cent mixture of ether and gasoline at 0° was precipitated by the addition of a large excess of gasoline. This fraction analyzed as follows:

No. 199. 0.2170 gm. substance required (Kjeldahl) 2.55 cc. of 0.1 N acid.
0.3150 " " : (fusion) 0.0356 gm. $Mg_2P_2O_7$.
0.1054 " " : (Carius) 0.0778 " AgBr.
Found. N 1.64, P 3.15, Br 31.41.

Bromo Acids from the Mixed Bromolecithins.

Hexabromostearic Acid.—40 gm. of the crude mixture of brominated lecithin were hydrolyzed for 8 hours with 10 parts of 10 per cent hydrochloric acid. The mixture of bromo and saturated fatty acids was separated by filtration, dissolved in ether, thoroughly washed with water, and dried. The total mixed acids weighed 28.5 gm. and had a bromine content of 29.03 per cent. (A mixture composed of 50 per cent stearic acid, 25 per cent hexabromostearic acid, and 25 per cent tetrabromostearic acid should have a bromine content of 29.16 per cent.) These crude acids were thoroughly extracted with gasoline and the insoluble bromo acid fraction was dissolved in methyl alcohol. From this solution, on cooling, a precipitate separated, which after recrystallization from methyl alcohol and thorough extraction with ether yielded 0.8 gm. of material melting at 180°, and giving the following analysis.

No. 191. 0.1045 gm. substance: 0.1120 gm. CO_2 and 0.0390 gm. H_2O.
0.1004 " " : (Carius) 0.1510 gm. AgBr.
Hexabromostearic acid, $C_{18}H_{30}O_2Br_6$.
Calculated. C 28.49, H 3.99, Br 63.26; m.p. = 180–181°.
Found. " 29.22, " 4.17, " 64.00.

When mixed with hexabromostearic acid obtained by brominating linolenic acid, no depression of the melting point was apparent.

Tetrabromostearic Acid.—The acids which remained dissolved in the methyl alcohol solution were precipitated with lead acetate, and the lead salts were suspended in toluene and decomposed. The addition of gasoline to the concentrated solution caused the immediate precipitation of an insoluble resinous material. On standing the liquor decanted from this gum deposited 2.5 gm. of the characteristic white crystals of tetrabromostearic acid. They gave the following analysis.

No. 192. 0.1032 gm. substance: 0.1354 gm. CO_2 and 0.0506 gm. H_2O.
    0.0744 "   "   : (Carius) 0.0956 gm. AgBr.
        Tetrabromostearic acid, $C_{18}H_{32}O_2Br_4$.
    Calculated.   C 36.01, H 5.38, Br 53.28; m.p. = 114°.
    Found.   " 35.77, " 5.48, " 54.69.

This material melted at 113–114°, and showed no depression of the melting point when mixed with tetrabromostearic acid prepared from linolic acid.

CONCLUSIONS.

1. Plant lecithin has been brominated.

2. From the bromolecithins a hexabromo and a tetrabromo derivative have been isolated.

3. This fractionation is regarded as evidence in favor of the theory viewing the ordinary lecithins as mixtures of several individual forms.

THE THIO-SUGAR FROM YEAST.

BY P. A. LEVENE AND HARRY SOBOTKA.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In 1912, Mandel and Dunham[1] discovered in the commercial yeast product "zymin" an adenine nucleoside of the elementary composition $C_{11}H_{15}N_5O_5$. They recognized the base as adenine but furnished little information as to the nature of the carbohydrate component.

Levene,[2] in 1924, in the course of work on brewers' yeast, incidentally isolated the substance discovered by Mandel and Dunham and corroborated their conclusions regarding the nature of the base. The sugar isolated from the nucleoside had the properties of a keto-hexose, but differed from all known ketohexoses.

Suzuki, as far back as 1914, described a base of the elementary composition $C_9H_{12}N_4O_4$. At the time of its discovery, he failed to recognize the glucosidic nature of the substance. Very recently, however, Suzuki, in cooperation with Odake and Mori[3,4] recognized the nucleosidic nature of the substance and made the most interesting discovery that the sugar contained sulfur in its molecule. In the German publication of their work, they consider as possible the first three of the following four structures. The fourth is contained in their Japanese publication.

I.

$$N = C - NH_2$$

Adenine.   Thio-methyl-pentose.
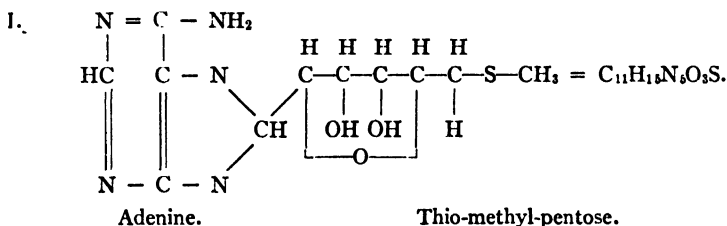
[1] Mandel, J. A., and Dunham, E. K., *J. Biol. Chem.*, 1912, xi, 85.

[2] Levene, P. A., *J. Biol. Chem.*, 1924, lix, 465.

[3] Suzuki, U., Odake, S., and Mori, T., *J. Agric. Chem. Soc. Japan*, 1924, i, No. 2.

[4] Suzuki, U., Odake, S., and Mori, T., *Biochem. Z.*, 1924, cliv, 278.

II.

$$N = C \cdot NH_2$$

HC C – N   H   H   H   H   H
                   C—C—C—C—C—CH₃.
           CH | OH OH | SH
                  └—O—┘
N – C – N

III.

$$N = C \cdot NH_2$$

HC C – N   H ┌—O—┐
                   C—C—C—C—C—CH₂SH = $C_{11}H_{18}N_4O_3S$.
           CH | H OH | H
                  └—O—┘
N – C – N

IV.

$$N = C \cdot NH_2$$

HC C – N   H   H   H   H   H
                   C—C—C—C—C—CH₃ = $C_{11}H_{18}N_4SO_2$.
           CH | OH OH | OH
                  └—S—┘
N – C – N
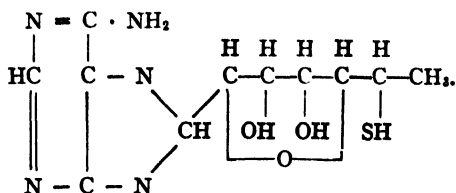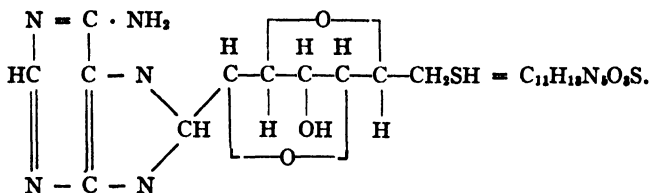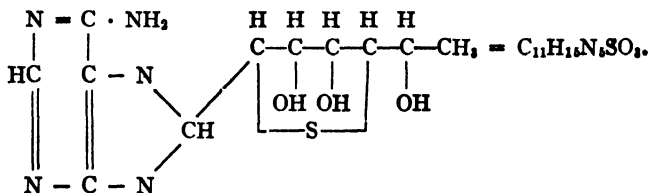
Suzuki and his coworkers, for some reason, did not associate their nucleoside with that of Mandel and Dunham. Had they done so, and had they taken into consideration the observations of Levene, they would have reduced the possibilities of the structure of their sulfo-sugar to a smaller number.

Since the molecular weight of sulfur is twice that of oxygen, it is evident that the carbon and hydrogen values for a hexose and for a sulfomethyl-pentose and the carbon and hydrogen and nitrogen values for their respective osazones are identical.

We have, therefore, tested the nucleoside prepared by one of us[2] and found that it contained the same proportion of sulfur as the substance of Suzuki. We have also analyzed the old osazones in our possession and found that they likewise possessed the requisite amount of sulfur. Furthermore, the para-bromophenylosazone described by Levene was prepared from the sugar after it had been exposed to the action of an excess of bromine during 96 hours. These data alone are sufficient to exclude first, the aldehydic structure accepted by Suzuki and his coworkers in all their formulæ and second, the presence of an —SH group as in figures (II) and (III).

The experiments previously reported by one of us have now been repeated. Our present results are identical with those already recorded. We have further extended them; namely, first, we prepared an acetyl derivative by the Behrend (pyridine) method. The substance was purified by distillation and had the composition of the triacetyl derivative. Therefore, formula (IV) of Suzuki is definitely excluded. Second, by distillation of the sugar with hydriodic acid we demonstrated the presence of either an $-OCH_3$ or an $-SCH_3$ group.

Since the sugar forms an osazone very readily, it is likely by analogy with other 2-keto-sugars that the $-SCH_3$ (or $-OCH_3$) group is not attached to carbon atom 1. Hence, the choice is reduced to the following two structures.

```
CH₂OH              CH₂OH
|   OH             |   OH
|  /               |  /
C------┐           C------┐
|      |           |      |
CHSCH₃ |           CHOCH₃ |
|      O           |      S
CHOH   |           CHOH   |
|      |           |      |
CH₂----┘           CH₂----┘

   V.                 VI.
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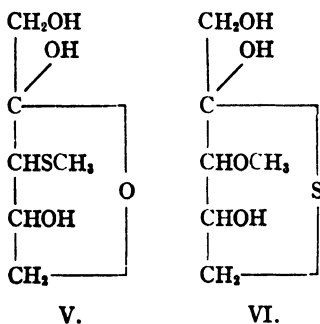
The position of the substituent group is for the present arbitrary, as it may be in position (4) as well.

The details of the configuration of the sugar will not be cleared up until the problem of Walden inversion receives an adequate solution.

EXPERIMENTAL PART.

The sugar used in these experiments consisted of a very thick syrup which was dried by removing the water by distilling at a pressure of 0.1 mm. The residue was taken up in alcohol and distilled as before. This operation was repeated several times, the ethyl alcohol being interchanged with methyl alcohol.

The final residue had the following rotation in methyl alcohol.

$$[\alpha]_D^{16} = \frac{+2.31° \times 100}{1 \times 5.51} = +41.9°$$

The substance analyzed as follows:

0.1183 gm. substance: 0.1744 gm. CO_2 and 0.0742 gm. H_2O.
$C_6H_{12}O_4S$.   Calculated.   C 39.96, H 6.72.
(180.17)   Found.   " 40.21, " 7.02.

For the estimation of the $-SCH_3$ group the substance was distilled with hydriodic acid in a slightly modified Zeisel apparatus, a solution of cadmium sulfate being used to absorb the hydrogen sulfide before the gases came in contact with the solution of silver nitrate, according to Kirpal and Bühn.[5]

0.1108 gm. substance: 0.1210 gm. AgI.
$C_6H_{12}O_4S$.   Calculated. $-SCH_3$ 26.13.
Found.   "   24.27.

Analysis of Osazone.
0.1075 gm. substance: 0.2372 gm. CO_2 and 0.0588 gm. H_2O.
0.1171 "   "   : (Carius) 0.0778 gm. $BaSO_4$.
0.0991 "   "   : (Dumas) 13.6 cc. nitrogen gas at $t = 23°C.$, $p = 757.5$ mm.
$C_{18}H_{22}N_4O_2S$.   Calculated.   C 60.30, H 6.19, N 15.64, S 8.93.
(358.21)   Found.   " 60.73, " 6.17, " 15.78, " 9.24.

Triacetyl Derivative.—5.0 gm. of the sugar in the form of a syrup dried in high vacuum (0.01 mm.) were treated with a solution containing equal parts of dry acetic anhydride in pyridine solution. The final product was purified by distillation. This brown heavy syrup was distilled at a pressure of 0.1 mm. Slight decomposition was noted at the beginning of the distillation but a fraction was obtained which boiled smoothly and constantly at a temperature of 170°C. The yield was 3 gm. The substance was found practically inactive.

This fraction analyzed as follows:

0.1036 gm. substance: 0.1778 gm. CO_2 and 0.556 gm. H_2O.
0.1618 "   "   : 0.1248 " $BaSO_4$.
$C_{12}H_{18}O_7S$.   Calculated.   C 47.04, H 5.92, S 10.47.
(306.21)   Found.   " 46.80, " 6.00, " 10.49.

[5] Kirpal, A., and Bühn, Th., *Ber. chem. Ges.*, 1914, xlvii, 1084.

THE MUCOPROTEINS OF THE SNAILS, HELIX ASPERSA AND HELIX POMATIA.

By P. A. LEVENE.

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The problem of the structure of complex proteins containing carbohydrates in their molecule is still a subject of discussion. The fundamental questions which have been debated during the last decades and which have been the subject of many controversies are concerned with the nature of the carbohydrate group present in the molecule of these substances. Since the discovery of mucoproteins or mucins, as all of them were termed originally, three mutually antagonistic views were advanced. Chronologically, they were the following: All mucoproteins are physical mixtures of a polysaccharide, resembling dextrin, and proteins. The polysaccharide was termed "animal gum" (Landwehr[1]). Through the experimental criticism of Hammarsten,[2] this view was modified by its author and in its final form it assumed a chemical union between the animal gum and the protein. The second view, of more recent date, was that the carbohydrate complexes of all mucoproteins were conjugated sulfuric acids of the type of mucoitin sulfuric acid. The third theory presented the structure of this group of substances in the form of a peptide or glucosidic linking between simple sugars (amino sugars) and proteins.

There were authorities who believed in the existence of representatives of each one of the three types of substances. Theoretically, every one of the three theories is possible and, therefore, the final decision will depend only on facts. However, the number of complex proteins containing carbohydrates in their molecules is very great and it will require much time and labor to analyze all of them.

A very critical and, for its time, a classical investigation into the

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[1] Landwehr, H. A., Z. physiol. Chem., 1882, vi, 74.

[2] Hammarsten, O., Arch. ges. Physiol., 1885, xxxvi, 412.

question of mucoproteins was carried out by Hammarsten 40 years ago. Hammarsten came to the conclusion that two types of proteins containing carbohydrates in their molecule were obtainable from *Helix pomatia*. One type is represented by the substances obtainable from the mucus secreted by these animals and by that extracted from the foot of the animal. These mucoproteins are characterized by their elementary composition which does not differ much from that of simple proteins. In distinction from these mucoproteins, he prepared one from the "protein glands" of these animals. The latter substance possessed a peculiarly low nitrogen content varying between 6 and 8 per cent, and readily yielded on extraction with water or better, on extraction with dilute alkali, a polysaccharide which he named "sinistrin."

No further work on the mucoproteins of these animals was recorded until very recently. Meanwhile the theory of the animal gum seemed to have been entirely discredited, on the one hand, by the failure of Folin in Hammarsten's laboratory to prepare nitrogen-free polysaccharides from several mucoproteins and, on the other hand, by the successful isolation of mucoitin and chondroitin sulfuric acids from many mucoproteins which was accomplished in the laboratories of Hofmeister and of the present writer, in cooperation with López-Suárez particularly.

In the year 1920, for the first time after the date of Hammarsten's publication, reference was made to the carbohydrate complex obtainable from the proteins of *Helix pomatia*. Schmiedeberg[3] in a very comprehensive publication on mucoproteins made a statement that from the proteins of *Helix pomatia* a nitrogen-free polysaccharide is obtainable which has the structure of a glucosan-pentosan.

Thus it is seen from this brief review that the work on the mucoproteins of *Helix pomatia* had a great influence on our conception of the structure of mucoproteins in general. Hence, it seemed expedient to test the old conclusions by more modern methods.

Every new work on this problem, of course, has the benefit of the experience of Hammarsten which led to a differentiation between the mucoproteins derived from the mucus of these animals and the substances obtained from the foot, on the one hand, and that obtained

[3] Schmiedeberg, O., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 44.

from the protein glands, on the other. It is unfortunate that the task of dissecting the glands of the animals is too difficult to permit its being carried out on a large scale. The present work was done on a larger scale than that of the older investigators; nevertheless the available material was not sufficiently large to permit as accurate an investigation as is required by modern standards.

Notwithstanding the many shortcomings, the results obtained in the course of the present work are sufficient to warrant several very definite conclusions. First, that the mucoprotein of the mucus belongs to the group derived from mucoitin sulfuric acid. A substance having the properties of the latter was isolated in a state free from protein and perfectly intact in every respect save for a loss of some of its sulfuric acid radicle. It gave on partial hydrolysis a disaccharide (mucosin), and after complete hydrolysis, there were obtained chitosamine and a volatile fatty acid in a proportion equivalent to one acetyl group for each atom of nitrogen. On distillation with hydrochloric acid, it gave furfural. When mucosin was treated in the same way the yield of furfural was equal to that required by the theory for equal proportions of glucuronic acid and of chitosamine. Whether the "uronic" acid was actually glucuronic, or galacturonic, or some other acid of this type was as yet not ascertained.

The present substance, like all other mucoitin sulfuric acids, did not reduce Fehling's solution directly, but did so after hydrolysis with mineral acids. It gave a positive test for glucuronic acid with naphthoresorcinol.

Very characteristic for the substance is its solubility. Like all other mucoitin sulfuric acids, it is insoluble in all organic solvents including glacial acetic acid. It is insoluble in water and in alkalies and is soluble in strong mineral acids. In these solutions the substance was dextrorotatory.

On the basis of solubility the present mucoitin sulfuric acid belongs to the subgroup A, of which the substance derived from "funis mucin" is a representative member.

Thus, the general characteristics of the mucoitin sulfuric acid derived from the mucus of the snails are quite clear and they confirm the conclusions expressed in previous publications that mucoproteins secreted by mucous membranes are derivatives of mucoitin sulfuric

acid. The interesting point attached to the mucoitin sulfuric acid obtained from the smails is its insolubility in water. Previously, substances of this type were obtained only from certain forms of connective tissue structures. Since the mucoitin sulfuric acid derived from the snails was prepared by a much simpler process than those which led to the soluble forms of mucoitin sulfuric acid, it is suggestive that the soluble mucoitin sulfuric acid is a product of the insoluble form. This suggestion is only tentative.

Somewhat more complex is the problem of the chemical structure of the polysaccharides obtained from the tissues of the snails. It was stated above that Hammarsten was the first to have pointed out that the carbohydrate obtained from the mucoprotein of the foot differed from that prepared from the body of the snail; the first having the properties of the carbohydrate radicle characteristic for other mucoproteins, the second being a distinctive substance. Our observations in certain respects are in harmony with those of Hammarsten. The complex carbohydrate prepared by us from the mucoprotein of the foot of the snails resembled, in the main, the analogous substance prepared from the mucus. The substance prepared from the mucoprotein of the foot, however, contained a small proportion of a different polysaccharide, which is more abundant in the bodies of the snails. As was mentioned above, Hammarsten discovered the pclysaccharide and referred to it as "sinistrin." We believe that the material prepared by Hammarsten was purer than ours as we did not attempt the extirpation of the gland with anatomical accuracy. The polysaccharide prepared by us was sparingly soluble in water but readily soluble in strong mineral acids and also, to some extent, in alkalies. In the majority of cases the solution was too opaque to permit a very dependable reading of its optical rotation. Whenever it was possible to take the rotation it was found to be to the right, and on a few occasions, the substance was inactive. The optical behavior alone was sufficient to indicate that the substance was not homogeneous. Furthermore, the substance contained small quantities of nitrogen and sulfur, which indicated that it contained a small proportion of mucoitin sulfuric acid. On hydrolysis with dilute mineral acids, the substance gave a maximum yield of about 60 per cent of a monosaccharide, calculated on the basis of glucose. Besides, on distillation

there was obtained between 20 and 30 per cent of acetic acid. The most interesting point in connection with the polysaccharide was the nature of the hexose. This was found to be that of galactose. The configuration of the hexose was suggested first by the phenylosazone prepared from it. This consisted of small platelets, was dextro-rotatory, showing, with time, a decline in the numerical value of the rotation. This property is peculiar among hexoses only to phenyl-galactosazone and to phenylgulosazone. Besides, the melting point of the osazone was 195°C., (uncorrected) which is characteristic for galactosazone. On oxidation with nitric acid, mucic acid was formed, which was identified by its melting point (m.p. 215°C.), by the absence of optical activity, and by its elementary composition.

Thus, the substance seems to be a polysaccharide composed of galactose, or perhaps of acetyl galactose. It is the first of this kind observed in animal tissues. Galactosans, to the knowledge of the author, have been prepared only from plants in the form of gums. Thus the term animal gum may be well applied to this substance.

The question then arises whether this polysaccharide is actually a component of a "glucoprotein," as Hammarsten suggested, or is a substance which occurs in the tissues of the snails in a free state and is adsorbed to the protein during the precipitation. The latter possi-bility seems to be suggested by the analytical data on the so called "glucoprotein." Hammarsten and before him, Eichwald and others have found that this protein contained only 8 per cent of nitrogen. No other mucoprotein is known with a nitrogen content as low as the one just mentioned. It was therefore attempted to extract the poly-saccharide directly from the organs by means of hot water. The method of Pflüger and Nerking was used for separating the polysac-charide from the protein material. Under these conditions, the same polysaccharide was obtained as that from the so called "glucoprotein" of Hammarsten. It was hydrolyzed into galactose and a volatile acid (acetic). This observation was in a way surprising, as Hammarsten reported the isolation of glycogen from the tissues of the snail. In this respect the experience of Hammarsten was at variance with that of Landwehr. Since the identification and the isolation of glycogen is a very simple matter, it is possible that the presence or absence of glycogen is conditioned by the nutritional state of the animals. It

was fortunate for us that in the tissues of our animals, glycogen was completely absent, and this fact facilitated the isolation of the galactose polysaccharide. It is possible, however, that still another polysaccharide is present in the tissues. This possibility is suggested because frequently the osazone prepared from the products of hydrolysis was optically inactive and melted at 202°C. On the other hand, only mucic acid could, with certainty, be isolated from the products of nitric acid oxidation of the polysaccharide. It is unfortunate that the material was not accessible in quantities to permit a more thorough purification of the polysaccharide and of its products of hydrolysis.

<div align="center">EXPERIMENTAL.</div>

<div align="center">PART I.</div>

The work on *Helix aspersa* Müller var. *maxima* Taylor will be reported first in view of the fact that the material was available in larger quantities and therefore permitted more detailed analysis.

Mucoprotein from the Mucus.—This material was collected according to the suggestion of Hammarsten. The shell of the animal was opened and by rubbing the animal with a glass rod it was caused to secrete the mucus. The fresh secretion was transferred into 95 per cent alcohol and kept until a quantity sufficient for work (about 500 gm. of the moist mucoprotein) was collected. In order to facilitate the precipitation of the mucoprotein, the alcohol was acidulated with acetic acid. The mucoprotein was then separated from the alcohol in part by passing through cheese-cloth, in part by filtration. The precipitate was transferred into a fresh portion of alcohol which was refluxed for 2 to 3 hours. The mucoprotein was then freed from the alcohol at first by filtration and subsequently by passing through a hydraulic press. The fairly dry cake was minced in a meat chopper. This material contained a considerable quantity of lime salts and, in order to remove the latter, it was suspended in large quantities of hot water containing 1 or 2 per cent of acetic acid and the mixture was turbinated. The water was renewed every 2 hours and the extraction continued until the suspended material on incineration left only a very small mineral residue. The suspended material was finally washed with pure water, again filtered, and passed through a hydraulic press.

Mucoitin Sulfuric Acid.

The mucoitin sulfuric acids can be prepared from this material in several ways. Each has its own advantages and the choice of the method should be determined by the purpose for which the material is needed.

First Process.—By this process, the mucoitin sulfuric acid is obtained in the most unaltered state. About 500 gm. of the mucoprotein, still moist, are taken up in 1000 cc. of a 5 per cent solution of sodium hydroxide and the mixture is placed in a shaking machine. After a short interval the mucoprotein is dissolved into a homogeneous viscous fluid. The shaking is continued for 48 hours. At the end of that time the viscous fluid is transferred into twice its volume of 95 per cent alcohol. A precipitate is then formed which is removed by centrifugalization. It is washed repeatedly with 95 per cent alcohol. The precipitate is then suspended in 800 cc. of 5 per cent aqueous sodium hydroxide and again shaken for 24 hours. At this phase the sodium salt of the mucoitin sulfuric acid appears in the main in the form of a suspension. At the end of the 24 hours the mixture is transferred into twice its volume of 95 per cent alcohol. The precipitate is separated from the fluid by centrifugalization. This material is practically insoluble in water. It has a gelatinous character and still gives a positive biuret test, which undoubtedly is due to the soluble protein which adheres mechanically to the mucoitin sulfuric acid. In order to remove the traces of the adhering soluble protein, the material is suspended in 5 per cent aqueous sodium hydroxide and placed in the shaking machine for an hour. After that time the mucoitin sulfuric acid is sedimented by centrifugalization. The alkali is replaced by distilled water and the suspension again is placed in a shaking machine. The latter operation is repeated as long as the mucoitin sulfuric acid gives a positive biuret test. The final product is biuret-free but still contains some calcium. In order to remove the latter, hydrochloric acid is added very cautiously to a suspension of the mucoitin sulfuric acid in water until the mixture reacts acid to Congo red. The suspension is then placed in a shaking machine for about $\frac{1}{2}$ hour. The dilute hydrochloric acid is replaced by distilled water and the shaking with fresh portions of distilled

water is repeated until the washings no longer contain chlorine ions. The final product is dried by treatment with alcohol and ether. The average yield from 500 gm. of moist mucoprotein is from 15 to 18 gm. of mucoitin sulfuic acid.

Second Process.—This process is much less laborious and time-consuming but has the disadvantage that it leads to a smaller yield and to a product which contains less sulfur than the substance obtainable by the first process.

The mucoprotein is taken up in twice its weight of 5 per cent solution of sodium hydroxide and placed on a water bath for 3 hours. The clear solution is then cooled and neutralized with acetic acid. A precipitate consisting chiefly of protein material is formed and removed by centrifugalization. From the supernatant liquid the mucoitin sulfuric acid is precipitated by means of basic lead acetate and ammonia. This precipitate is freed from supernatant liquid by centrifugalization. The precipitate is washed several times with water and is then taken up in enough concentrated hydrochloric acid to convert all the lead into its chloride. The lead chloride is removed by centrifugalization and the supernatant liquid is poured into a large excess of glacial acetic acid. The mucoitin sulfuric acid settles out in the form of a flocculent precipitate. This again is freed from the supernatant liquid by centrifugalization. It is taken up in a little water and precipitated by means of alcohol. By this process protein-free mucoitin sulfuric acid can be prepared in about 48 hours. The maximum yield obtainable by this process was 10 gm. out of 500 gm. of moist mucoprotein.

A substance which as regards its sulfur content occupies a position intermediate between the other two is obtained when the lead salt is suspended in water containing barium acetate and a stream of hydrogen sulfide gas is passed through the mixture. The filtrate is then concentrated to a very small volume and is poured into a large excess of glacial acetic acid. The product obtained in this way is taken up in water and reprecipitated with alcohol.

Properties of the Mucoitin Sulfuric Acid.—As was already stated by each one of the above methods, a biurèt-free substance is obtainable. It is very little soluble in water, in aqueous alkalies, or in dilute mineral acids. It is soluble in concentrated mineral acids giving a slightly opalescent solution. The substance does not reduce Fehling's solution

but shows a strong reduction after preliminary hydrolysis. The substance gives with naphthoresorcinol a very strong test for glucuronic acid. The best samples prepared by the first process contained only half the theoretical amount of sulfuric acid; on the other hand, after refluxing for 1 hour over a free flame with a solution of 20 per cent of oxalic acid, a product is obtained which still contains 1 per cent of sulfur.

Composition of the Various Samples of Mucoitin Sulfuric Acid.

Sample I prepared by the first process:

0.1000 gm. substance required for neutralization (Kjeldahl) 1.90 cc. 0.1 N acid. N 2.66.

0.2048 gm. substance: 0.0350 gm. $BaSO_4$. S 2.34.

Sample II prepared by the second process:

0.0924 gm. substance: 0.1370 gm. CO_2 and 0.0468 gm. H_2O. C 40.43, H 5.66.

0.1626 gm. substance neutralized (Kjeldahl) 3.0 cc. of 0.1 N acid. N 2.58.

0.1635 gm. substance: 0.0136 gm. $BaSO_4$. S 1.14.

Sample III prepared according to the third process:

0.1000 gm. substance neutralized (Kjeldahl) 3.50 cc. of 0.1 N acid. N 4.90.

0.2590 gm. substance: 0.0312 gm. $BaSO_2$. S 1.65.

$C_{28}H_{48}O_{29}N_2S_2$. Calculated.   C 35.72, H 5.18, N 2.99, S 6.82.

      Found. Sample I.   " 2.66, " 2.34.

            "   II.   " 40.43, " 5.66, " 2.58, " 1.14.

            "   III.   " 4.90, " 1.65.

Mucosin.

It was pointed out in previous publications on mucoitin sulfuric acids that mucosin was much less stable than chondrosin, and that great caution must be exercised not to permit the hydrolysis to pass beyond the stage of mucosin formation. To avoid the latter danger, it was attempted to hydrolyze the mucoitin sulfuric acid by means of oxalic acid. 10 gm. of the substance were taken up in 200 cc. of a 20 per cent solution of oxalic acid and refluxed over a free flame for 1 hour. At the end of that time the heavy floccules of the mucoitin sulfuric acid disappeared leaving only a fine white precipitate consisting, in the main, of calcium oxalate. The solution was practically colorless. It was freed from the sediment by centrifugalization and was poured into 5 volumes of a mixture consisting of equal parts of alcohol and ether.

In this manner a substance was obtained which was more soluble than the original substance. The substance had 50 per cent of its

nitrogen in the form of amino nitogen and still contained 1 per cent of sulfur. The yield of the substance was 5 gm. from 10 gm. of the original material. These 5 gm. were then dissolved in 100 cc. of 10 per cent hydrochloric acid, and refluxed for $\frac{1}{2}$ hour on a boiling water bath. The solution was then concentrated under reduced pressure to 5 cc. and poured into 600 cc. of a mixture of equal parts of alcohol and ether. The material obtained after this operation differed only little from the original. The proportion of amino to total nitrogen had increased to about 60 per cent. The yield from 5 gm. was 4.8 gm. This material was then dissolved in 50 cc. of 20 per cent hydrochloric acid and treated as before; 1.2 gm. of mucosin were obtained. The substance was slightly colored and could not be used for optical measurement. 0.050 gm. of the substance had the reducing power of 0.027 gm. of glucose.

The substance contained 4.69 per cent of total nitrogen and 4.02 per cent of amino nitrogen. 0.400 gm. of the substance gave on distillation 0.065 gm. of phloroglucide of furfural, which corresponds to 50 per cent of glucuronic acid.

Thus the substance had the properties of mucosin but was as yet crude. Unfortunately, further purification was impossible because of the lack of material.

Chitosamine.

5 gm. were taken up in 25 cc. of 20 per cent hydrochloric acid to which 2 gm. of barium chloride and 1 gm. of stannous chloride had been added and refluxed over a free flame. The solution was then freed from barium and from tin and concentrated under reduced pressure. Chitosamine hydrochloride crystallized out. The substance turned dark above 200°C., but had no melting point. It analyzed as follows:

No. 144.　0.0100 gm. substance: 1.20 cc. nitrogen gas (Van Slyke).
$$C_6H_{13}O_5N \cdot HCl. \quad \text{Calculated.} \quad N \ 6.51.$$
$$\text{Found.} \quad \text{"} \quad 6.73.$$

The optical rotation of the substance was

Initial.	Equilibrium.
$[\alpha]_D^{20} = \dfrac{+0.98° \times 100}{1 \times 1} = +98°$	$[\alpha]_D^{20} = \dfrac{+0.76° \times 100}{1 \times 1} = +76°$

Furfural.

The distillation of furfural was conducted under the usual conditions. The yield of the phloroglucide was much below that required by theory. One of the reasons, however, might have been the fact that only small quantities of material were available for these experiments. On the other hand, from mucosin, the yield of the phloroglucide of furfural agreed with that required by the theory. Thus, 0.5000 gm. of the crude product yielded 0.035 gm. of phloroglucide of furfural, which corresponds to 0.100 gm. or 20 per cent of glucuronic acid, whereas the theory requires 40 per cent.

Estimation of the Volatile Fatty Acids.

The volatile fatty acids were estimated in two ways. In one case, the material was suspended in an excess of barium hydroxide and refluxed over a free flame in an apparatus protected against the absorption of carbon dioxide from the air. The operation was continued for 4 hours at the end of which time it was interrupted, and the material allowed to cool. The mixture was then made acid to Congo red by means of sulfuric acid and distilled. The distillate was received in 0.1 N alkali protected against the absorption of carbon dioxide from the air.

Either 1 or 0.500 gm. of the material was used for distillation. Calculated for acetic acid, the yields varied between 10 and 15 per cent. The yield required by theory for free chondroitin sulfuric acid is 12.6 per cent. The small amount of available material did not permit the isolation and the identification of the acid.

Polysaccharide from the Mucoproteins of the Foot of the Snails.

From the foot of the snails the mucoprotein was extracted according to the directions of Hammarsten. The minced organs were extracted with a 0.1 per cent solution of sodium hydroxide, the solution centrifugalized, and the mucoprotein precipitated from the supernatant liquid by means of acetic acid. The precipitate thus formed was redissolved and reprecipitated several times. The final product contained 14 per cent of nitrogen. The final precipitate of the mucoprotein was refluxed with 95 per cent alcohol for several hours, then taken

up in alkali, and further treated exactly according to the second process described above and the product obtained from it was undoubtedly a mixture of mucoitin sulfuric acid with the polysaccharide. The proportion of the former was undoubtedly very large. This is quite natural since the dissecting of the foot from the body was not carried out with anatomical accuracy. The composition of one of the samples obtained by this process was the following.

C 40.39, H 6.19, N 3.58, S 0.48.

The substance gave a negative biuret test and with naphthoresorcinol a strong test for glucuronic acid.

Polysaccharide from the So Called "Glucoprotein."

The so called "glucoprotein" was prepared from the bodies of the snails in exactly the same manner as the mucoprotein from the foot. It contained 12 per cent of nitrogen. The carbohydrate was isolated by the second process employed for the preparation of mucoitin sulfuric acid from the mucoprotein of the mucus. The final product obtained in this manner was a slightly grayish white powder. It was never completely soluble in water. The solution was practically always opaque. The substance was completely soluble in hydrochloric acid of specific gravity 1.19 and from this solution it is precipitated by means of alcohol. In hydrochloric acid solution the substance is slightly dextrorotatory. Some samples appeared entirely inactive. The substance always contained small proportions of nitrogen and sulfur, though the biuret test was apparently negative. No perceptible test for glucuronic acid could be obtained with naphthoresorcinol; also with orcinol the test was negative. The yield of furfural on distillation was negligible. The small proportions of nitrogen and sulfur (when present) were undoubtedly due to insignificant admixtures of mucoitin sulfuric acid. The substance gave with iodine a negative test for either starch or dextrin.

Samples were obtained containing as little as 0.3 to 0.6 per cent of nitrogen and contained no sulfur.

The sample which was used for the experiments described below had the following composition. The purer samples were too small for further work.

Sample No. 598.   C 42.61, H 6.38, N 0.98, S 0.67.

Hydrolysis of the Polysaccharide.

1 gm. of the substance was hydrolyzed by refluxing over a free flame with 100 cc. of 4 per cent sulfuric acid. The resulting perfectly colorless solution was dextrorotatory ($[\alpha] = +0.32°$) and reduced Fehling's solution equivalent to 0.586 gm. of glucose.

From this solution an osazone was obtained which crystallized from pyridine and alcohol. It crystallized in bright platelets melting at 197° (uncorrected).

The rotation of the osazone in pyridine and alcohol solution ($c = 1$ per cent; $l = 50$ mm.).

Initial.	Equilibrium.
$\alpha_D^{20} = +0.35°$	$\alpha_D^{20} = +0.16°$

The rotation and the direction of the mutarotation of the substance suggested that it was a phenylgalactosazone.

From samples which contained more impurities the osazone was inactive and in such cases, the melting point was somewhat higher; namely, 202°C.

Nitric Acid Oxidation.

3 gm. of the polysaccharide were hydrolyzed by refluxing for 8 hours over a free flame in 150 cc. of 10 per cent nitric acid. The solution was then concentrated to 30 cc., an equal volume of nitric acid of specific gravity 1.4 was added, and the solution was allowed to stand overnight and then rapidly oxidized. From this material 0.500 gm. of mucic acid was obtained. The substance was optically inactive, melted at 215°C., and had the following composition.

0.1000 gm. substance: 0.1252 gm. CO_2 and 0.0442 gm. H_2O.
$C_6H_{10}O_8$. Calculated. C 34.22, H 4.80.
Found. " 34.14, " 4.95.

Acetyl Estimation.

1 gm. of the material was used for acetyl estimation by the barium hydroxide hydrolysis and subsequent distillation of the hydrolysate acidulated with sulfuric acid as described in an earlier section of this

paper. The yield was 0.200 gm. of acetic acid which was identified as the silver salt. For analysis it was recrystallized from water.

0.050 gm. substance: 0.0324 gm. Ag.

$C_2H_3O_2Ag$.   Calculated.   Ag. 64.31.
Found.   " 64.80.

Polysaccharide Obtained Directly from the Bodies of the Snails.

The initial stages of the isolation of this polysaccharide were those recommended by Pflüger and Nerking for the isolation of glycogen. The minced material was allowed to stand on the boiling water bath for 3 hours with 2 volumes of hot water. The mixture was then made to contain 3 per cent of potassium hydroxide and 8 per cent of potassium iodide and to the mixture alcohol was added as long as it continued to produce precipitation. The precipitate was collected by centrifugalization and was washed several times with a 50 per cent solution of alcohol containing 3 per cent of alkali and 8 per cent of potassium iodide.

Further purification was very tedious. The crude material was dissolved in hydrochloric acid of specific gravity 1.19, care being taken to avoid warming the solution. The solution (cooled in an ice and salt mixture) was centrifugalized and the supernatant liquid was poured into alcohol. The precipitate was then centrifugalized and washed with alcohol until all the free mineral acid was removed. The precipitate was then suspended in water and reprecipitated by alcohol. In this manner the still adhering traces of hydrochloric acid were removed completely. The product obtained in this manner was a mixture of the new polysaccharide and mucoitin sulfuric acid.

For the separation of the two, advantage is taken of the differences in their solubilities. The mucoitin sulfuric acid is the more insoluble product, and the polysaccharide is more soluble. The product just described was taken up in water and centrifugalized. From the supernatant liquid, the polysaccharide was precipitated by means of alcohol. The insoluble fractions could be purified by taking up in a little hydrochloric acid so that only part went into solution. The insoluble part contained 3.15 per cent of nitrogen and 1.5 per cent of sulfur.

The polysaccharide was again dissolved in water and centrifugalized, and the supernatant liquid was precipitated with alcohol.

The final product had the same properties as the polysaccharide obtained from the so called "glucoprotein." It also contained a small admixture of mucoitin sulfuric acid which could undoubtedly be entirely removed if sufficient material were available.

Sample I. 0.0925 gm. substance required (Kjeldahl) 0.65 cc. 0.1 N alkali.
0.2041 gm. substance: 0.0100 gm. $BaSO_4$ (Carius).
0.1052 " " : 0.1644 gm. CO_2 and 0.0600 gm. H_2O.
Found. C 42.61, H 6.38, N 0.98, S 0.67.

Purer samples were obtainable when the hot aqueous extracts of the bodies were removed by centrifugalization and only the residues were treated as above. The product obtained in this manner contained practically no sulfur and only minimal proportions of nitrogen, as is illustrated by the following two samples.

No. 572. 0.1000 gm. substance required for neutralization (Kjeldahl) 0.20 cc. 0.1 N alkali. N 0.28.

No. 580. 0.1000 gm. substance required for neutralization (Kjeldahl) 0.30 cc. 0.1 N alkali. N 0.42.

1 gm. of the substance containing 0.9 per cent of nitrogen was hydrolyzed with 100 cc. of a 4 per cent solution of sulfuric acid. The solution was dextrorotatory ($\alpha = +0.60°$ in a 100 mm. tube) and contained 0.6287 gm. of reducing sugar, calculted on the basis of glucose. (The volume of the solution was 87 cc.) The osazone prepared from it was optically inactive and had a melting point of 202°C.

No. 614. 0.0626 gm. substance: 8.70 cc. of nitrogen gas at 28°C. and 756.2 mm.
$C_{18}H_{22}O_4N_4$. Calculated. N 15.63.
Found. " 15.69.

Oxidation with Nitric Acid.

3 gm. of the substance were hydrolyzed by refluxing over a free flame for 8 hours with 10 per cent nitric acid and the operation was repeated as in the above described experiment. The yield of crude mucic acid was 0.565 gm. For analysis it was twice recrystallized out of water. It was optically inactive, had a melting point of 215°C., and had the following composition.

0.1040 gm. substance: 0.1298 gm. CO_2 and 0.0458 gm. H_2O.
$C_6H_{10}O_8$. Calculated. C 34.22, H 4.80.
Found. " 34.03, " 4.91.

It is possible that the mother liquor from mucic acid contained saccharic acid. The mother liquor from mucic acid was rendered alkaline by means of potassium hydroxide and the solution was then acidulated with acetic acid. On addition of a little alcohol and subsequent scratching the walls of the beaker with a glass rod a crystalline deposit began to form.

The acid potassium salt was then converted into the silver salt. Two fractions were obtained which were analyzed without recrystallization. One (the major portion) contained 54.0 per cent of silver, the other, 62.0 per cent of silver. The silver salts of saccharic and anhydrosaccharic acids contain 50.95 and 53.2 per cent of silver respectively. Silver oxalate contains 71.0 per cent. Since the mother liquor from the mucic acid did contain some oxalic acid,˙ it is not possible for the present to interpret the significance of these silver salts. If saccharic acid were present, it may be derived from chitosamine, since the polysaccharide still contained a small impurity of mucoitin sulfuric acid.

PART II.

Helix pomatia.

This snail was available in smaller quantities. However, practically all that was observed on the Helix aspersa was noted also on Helix pomatia. The mucoprotein from the mucus contained a mucoitin sulfuric acid, the so called "glucoprotein." However, in this case, no attempt was made to isolate the polysaccharide directly from the bodies of the snails.

The procedures for isolation and analysis were exactly the same as described in the first part of the paper and will therefore be omitted in this place. Only the analytical results will be reported.

Mucoitin Sulfuric Acid Obtained from Mucoprotein of the Mucins.

The substance was obtained by the two procedures described in the first part.

One of the samples obtained by the first process was converted into barium salt and analyzed as follows:

0.2000 gm. substance neutralized (Kjeldahl) 3.65 cc. 0.1 N acid. N 2.32.
0.2086 gm. substance : 0.0762 gm. BaSO₄. S 5.37.
0.2054 "   "   : 0.0294 "   "   Ba 9.01.

$C_{28}H_{44}O_{29}N_2S_2Ba_2$. Calculated. N 2.32, S 5.30, Ba 22.70.
                     Found.   " 2.32, " 5.37, " 9.01.

The composition of the products varied from one experiment to the other.

Chitosamine.

The chitosamine obtained from this material had the following composition.

0.020 gm. substance: (Van Slyke) 2.35 cc. nitrogen gas at 25°C. and 769.9 mm.

$C_6H_{13}O_5N \cdot HCl$. Calculated. N 6.51.
                Found.   " 6.61.

The optical rotation of the substance in 2.5 per cent hydrochloric acid was

Initial.

$$[\alpha]_D^{25} = \frac{+0.96° \times 100}{1 \times 1} = +96°$$

Equilibrium.

$$[\alpha]_D^{25} = \frac{+0.76° \times 100}{1 \times 1} = +76°$$

The mucoitin sulfuric acid gave on distillation 8 per cent of the phloroglucide of furfural, which corresponds to about 24 per cent of glucuronic acid. The acetyl value corresponded to 15 per cent. The acetic acid was not isolated.

Polysaccharide from the So Called "Glucoprotein."

The polysaccharide was optically inactive and had the following composition. The purest samples contained 0.3 and 0.4 per cent of nitrogen. The larger sample had the following composition.

C 36.88, H 5.09, N 1.2, S 0.8, and 8 per cent ash.

1 gm. on hydrolysis gave in 84 cc. a dextrorotation of $\alpha_D = +0.46°$ and reducing power equivalent to 0.560 gm. of glucose. The phenylosazone prepared from it had the appearance of a galactose. It had in pyridine-alcohol solution ($c = 1$ per cent, $l = 50$ mm.), an initial rotation of $\alpha_D = +0.10°$ and an equilibrium rotation of $\alpha_D = +0.10°$.

0.0748 gm. substance: 10.20 cc. nitrogen gas at 26.0°C. and 7.53 mm.
$C_{18}H_{22}O_4N_4$.   Calculated.   N 15.63.
Found.   " 15.45.

1 gm. of the substance was oxidized with nitric acid and gave about 0.200 gm. of mucic acid. The substance was optically inactive and had a melting point of 215°C.

CONCLUSIONS.

1. The carbohydrate radicle isolated from the mucoproteins of the mucus of *Helix aspera* and *Helix pomatia* belongs to the group of mucoitin sulfuric acid. On partial hydrolysis the polysaccharides yield the disaccharide mucosin. From the product of complete hydrolysis there were isolated sulfuric acid, chitosamine, and a volatile fatty acid. On distillation with hydrochloric acid, the mucosin yielded furfural in a quantity required by the theory of the structure of this disaccharide.

2. From the bodies of the snails a substance is obtainable which may be regarded as animal gum and which consists largely of a polygalactose, perhaps of an acetylated polygalactose.

3. From the bodies of the snails by mere extraction with water and by further treatment by the process of Pflüger and Nerking, an identical polysaccharide can be isolated.   .

PROTEIN FILMS ON COLLODION MEMBRANES.*

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I.

INTRODUCTION.

It was observed by Loeb[1] that collodion bags which had been filled with gelatin solutions and then washed many times with water behaved differently from untreated collodion bags in experiments on the rate of diffusion of electrolytes and water through the collodion. This behavior seemed to indicate the existence of a film of protein adhering to the collodion. Loeb obtained similar results by treating collodion bags with solutions of other proteins, such as casein, egg albumin, blood albumin, edestin, and oxyhemoglobin. In the latter case the existence of the film was made evident by the persistence of a red coloration on the membrane. The film can be rendered visible in the case of other proteins by an experiment suggested by J. H. Northrop, which consists in placing a piece of the protein-treated and washed membrane in acetone; the collodion rapidly dissolves, leaving the insoluble protein as a bulky opaque residue. The present work was undertaken in order to study some of the variables on which this film formation depends.

II.

EXPERIMENTAL METHOD.

The proteins used were gelatin and egg albumin, purified[2] by the methods of Loeb and Sörensen, respectively. The membranes were flat disks of collodion, prepared from a solution made by diluting

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*Presented before the Leather and Gelatin Division of the American Chemical Society at Baltimore, April 8, 1925, and published here by permission of the Editor of the *Journal of the American Chemical Society.*

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 255, 577.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York, 1st edition, 1922; 2nd edition, 1924.

Merck's collodion (U. S. P. IX) with an equal volume of a mixture of 75 parts by volume of ether with 25 parts of 95 per cent alcohol. This gave a solution containing about 2 gm. of dry collodion in 100 cc. The membranes first used were prepared on a circular glass plate 10.1 cm. in diameter, levelled by being floated in a Petri dish full of mercury. 10 cc. of the collodion solution were allowed to flow from a pipette on to the glass plate, which was shielded by a paper cylinder[3] (enclosing also a small open vessel containing ether) for the few seconds required for the collodion to spread over the surface of the glass. The shield was then removed, the time noted, and the solvents were allowed to evaporate from the collodion for 15 minutes at room temperature (about 20°C.) At the end of this time the plate and membrane were put in water, and the membranes were kept in water until the following day, when they were used.

Each membrane was blotted between filter papers, rolled loosely, and put into a Pyrex test-tube with 25 cc. of a protein solution. The test-tubes were tightly closed by rubber stoppers and rotated end over end for about 16 hours in a water thermostat at 37°C. ±0.02°. The amount of protein adhering to each membrane was determined by removing the membranes, washing each by stirring a few times in 300 cc. of water at 37°C., drying for 1 hour in an oven at 100°C., and weighing the dry membrane with its adherent protein. At the same time two or three untreated membranes were similarly dried to obtain the dry weight of the collodion alone. The weights of membranes from the same lot always agreed to 1 or 2 mg., being within 5 mg. of 0.200 gm. Membranes dried 2, 4, and 21 hours differed in weight by less than 1 mg. from those dried 1 hour. This method for determining the weights of protein was checked to about 2 per cent by dry weight determinations of gelatin solutions before and after shaking with the membrane, the gelatin samples being dried for 24 hours at 100°C.

<center>III.</center>

<center>EFFECT OF PROTEIN CONCENTRATION.</center>

Experiments were carried out by the above method with solutions of isoelectric gelatin and egg albumin of different concentrations.

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[3] Nelson, J. M., and Morgan, D. P., Jr., *J. Biol. Chem.*, 1923–24, lviii, 305.

When the egg albumin solutions were shaken with collodion membranes at 37°C., it was found that part of the protein was denatured and precipitated out.   Accordingly the egg albumin solutions were not shaken, but simply left in contact with the membranes at 37°C. Each membrane was cut in halves so that it might be completely submerged in the solution.   Some typical results are given in Fig. 1. The curves appear to have the general shape found in many types of adsorption experiments.   It was found, however, that the results
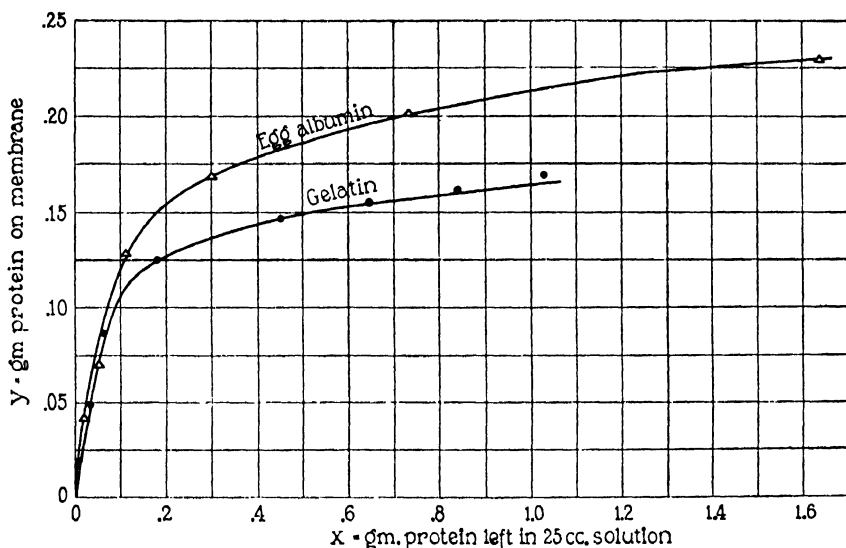
FIG. 1. Effect of protein concentration on the adsorption of gelatin and egg albumin by collodion membranes.

could not be fitted by the hyperbolic equation of Freundlich,[4] which is often referred to as the adsorption isotherm.   His equation has the form $y = kx^{\frac{1}{n}}$, where y is the amount of substance adsorbed by a fixed amount of adsorbent, x is the concentration of the solution at equilibrium, and k and n are constants to be determined from the experimental values of x and y.   The fact that this equation does not apply to the experiments of Fig. 1 is shown by Fig. 2, where the logarithms of the amounts adsorbed are plotted against the logarithms

[4] Freundlich, H., Kapillarchemie, Leipsic, 3rd edition, 1923, 151, 232.
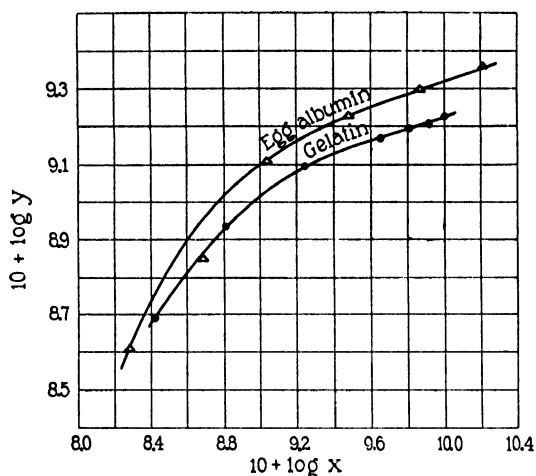
FIG. 2. Test of the application of Freundlich's equation, $y = kx^{\frac{1}{n}}$, to the data shown in Fig. 1. Since the curves are not straight lines, the equation does not apply to these experiments.
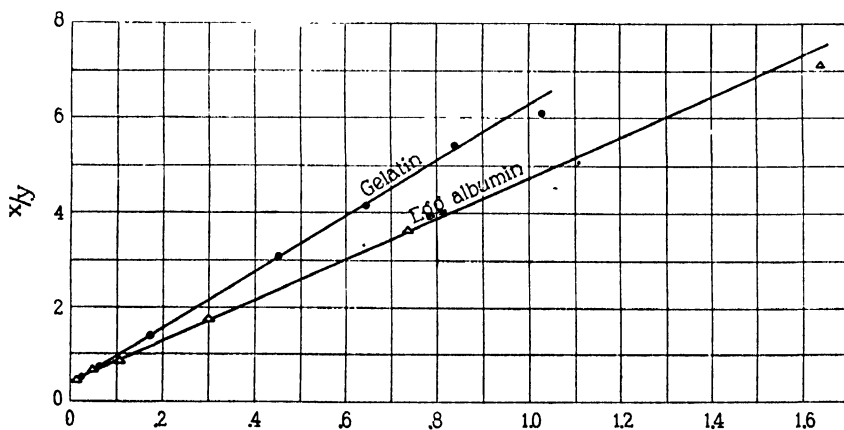
FIG. 3. Test of the application of Langmuir's equation, $\frac{x}{y} = \frac{1}{ab} + \frac{x}{b}$, to the data shown in Fig. 1. Since the curves are straight lines, the equation applies to these experiments.

of the final concentrations. If the equation applied, the points of each experiment should fall on a straight line.

Fig. 3 represents a test of the applicability to the same data of an

equation of hyperbolic type derived by Langmuir[5] for the adsorption of a gas on a plane surface. This equation has the form

$$ y = \frac{a\,b\,x}{1 + ax} \qquad \text{or} \qquad \frac{x}{y} = \frac{1}{ab} + \frac{x}{b} $$

where x and y have the same meaning as before and a and b are constants. In Fig. 3 the values of $\frac{x}{y}$ are plotted against those of x, and if the equation applies, the data should give straight lines. The figure shows that this is very nearly the case. The value of $\frac{1}{ab}$ is given by the intercept on the axis of ordinates, while the value of $\frac{1}{b}$ is the slope of the line. This equation of Langmuir predicts that in higher concentrations the amount is adsorbed, y, should reach a constant limiting value, b, while Freundlich's equation does not. In the experiments to be described below, in which smaller membranes were used, such limiting values were definitely obtained.[6]

<div align="center">IV.</div>

<div align="center">EFFECT OF HYDROGEN ION CONCENTRATION.</div>

The effect of hydrogen ion concentration on the removal of protein from solution was determined with solutions containing varying amounts of HCl or NaOH together with 0.5 gm. of gelatin or egg albumin in 25 cc. The weight of the protein film was obtained directly as outlined above, and the pH of each solution was determined with the hydrogen electrode at 25°C. The results are given in Fig. 4. In each case the amount of protein adhering to the membrane was greater at the isoelectric point than at any other pH.

The effect of different acids on the amounts of gelatin adhering to the collodion is shown in Fig. 5. The points obtained with CH_3COOH and H_3PO_4 are quite close to the curve for HCl, while the curve for H_2SO_4 is considerably higher.

[5] Langmuir, I., *J. Am. Chem. Soc.*, 1918, xl, 1368, 1384.

[6] It may be noted that an equation of the same form as this adsorption equation of Langmuir would result from the law of mass action as applied to a reversible combination between two substances, one at a fixed total concentration, the other, variable.
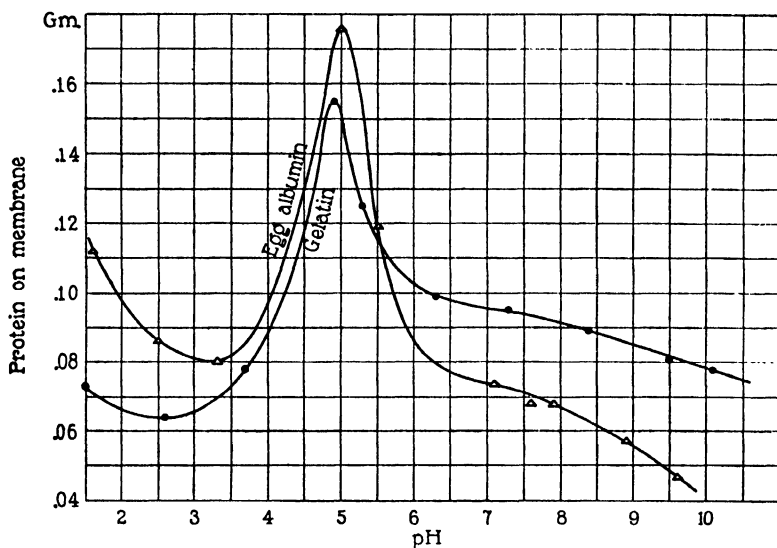
FIG. 4. Effect of pH on the adsorption of gelatin and egg albumin from 2 per cent solutions by collodion membranes.
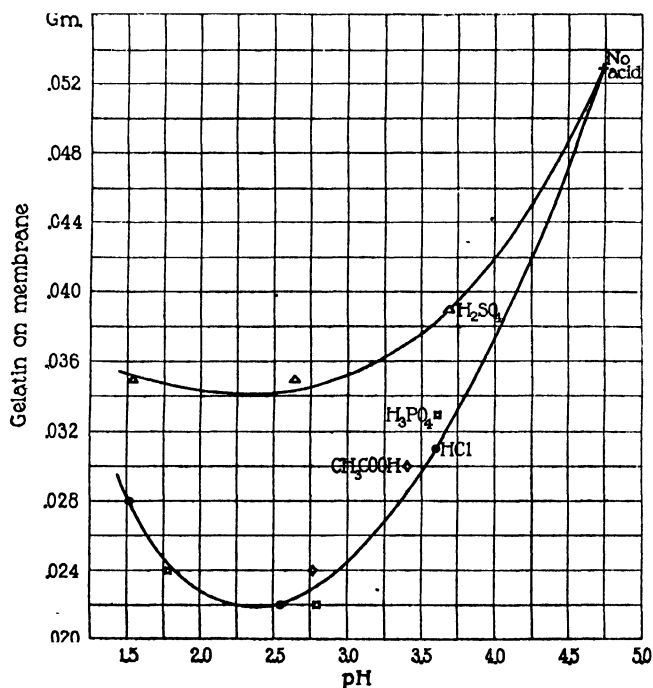
FIG. 5. Effect of different acids on the adsorption of gelatin from 2 per cent solutions by collodion membranes.

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Fig. 6 shows the effect of salt concentration on the adsorption of gelatin from 2 per cent solutions containing 0.02 M HCl, pH about 2.5. While the values obtained in low concentrations of salt are erratic, it is evident from the figure that each salt tended to increase the amount adsorbed up to the value obtained at the isoelectric point with no salt, Na_2SO_4 having more effect than NaCl. In this experiment the weight of the combined ash of the thirteen protein-coated membranes was found to be only 0.001 gm., which shows that the increased weight obtained was not due to adherent salt.
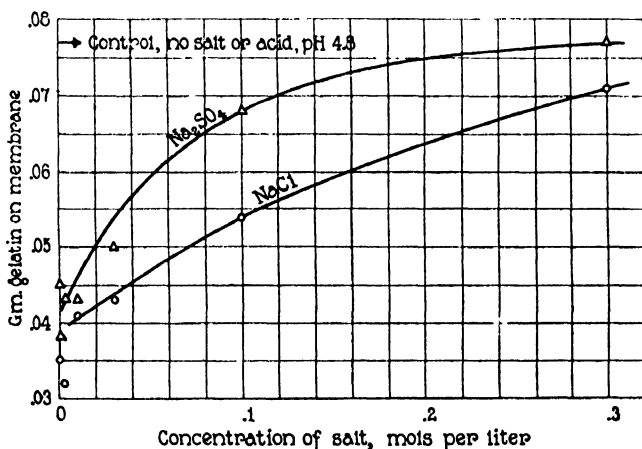
FIG. 6. Effect of salts on the adsorption by collodion membranes of gelatin from 2 per cent solutions in 0.02 M HCl (pH 2.5).

The curves in Figs. 4, 5, and 6 show that the amount of gelatin adhering to the collodion membranes was affected by the same variables which Loeb[2] found to affect the viscosity and other colloidal properties of gelatin solutions, but in an opposite direction. Fig. 7 gives a comparison of the effect of pH on the amount of adherent gelatin and on the fluidity of 2 per cent gelatin solutions which had been kept overnight at 37°C. The fluidity values, in c. g. s. units, were calculated from relative viscosity determinations made with Ostwald viscometers, by means of Bingham's[7] data for the absolute viscosity of water. Except for the high fluidities at low pH, which

[7] Bingham, E. C., Fluidity and plasticity, New York and London, 1922.

are probably due to hydrolysis of the gelatin, the two curves show a marked parallelism.[8]

The explanation for these results is still uncertain. The following suggestion may be tentatively made. If Loeb's explanation for the viscosity of gelatin solutions at 20° or 24° applies to solutions at 37°, then the effect of pH is to change the size of submicroscopic particles by affecting the degree of swelling conditioned by the Donnan equilib-
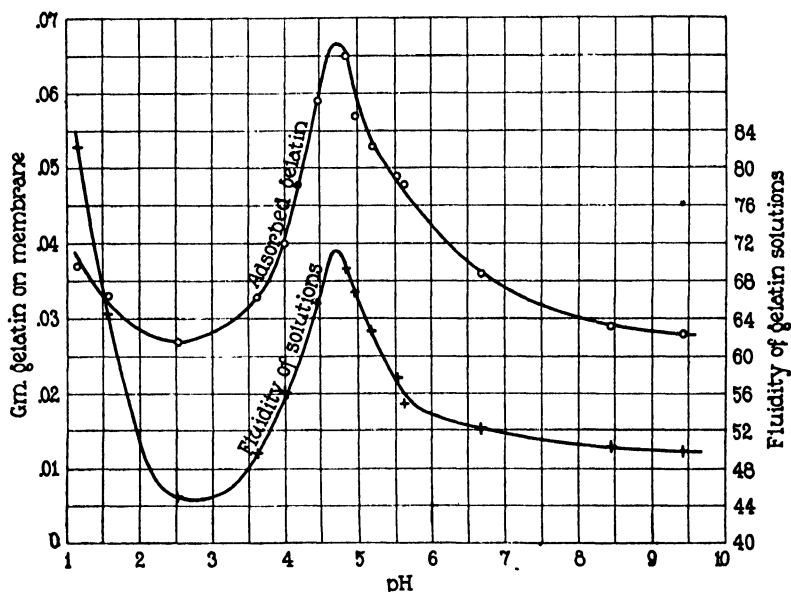
FIG. 7. Effect of pH on the fluidity of gelatin solutions and on the amount of gelatin adsorbed by collodion membranes.

rium. Accordingly when the viscosity is at a minimum the particles are smallest, and hence more particles can get in contact with the surface of the membrane and adhere. In the case of egg albumin the explanation is probably different, for with this protein Loeb found no marked effect of pH on viscosity. More experiments will be required before any explanation for these pH curves can be advanced with certainty.

[8] A coincidence of minimum adsorption with maximum viscosity in alkaline solutions has been reported (Abderhalden, E., and Fodor, A., *Kolloid- Z.*, 1920, xxvii, 49) in the case of the adsorption by charcoal of casein and a yeast protein. In acid solutions of the latter the addition of salt increased the adsorption.

V.

EFFECT OF PERMEABILITY OF MEMBRANES.

In experiments with membranes made on different days by the method described above, the results were not reproducible. This irregularity was found to be due to variations in the permeability of the membranes caused by variations in the temperature and humidity of the laboratory when the membranes were prepared. It was found that as the time of drying the membranes before immersion in water was increased, their capacity to adsorb gelatin decreased. Membranes which had been allowed to become very impermeable, by being dried for 1 hour or more, did not take up any measurable quantity of gelatin.

In order to obtain membranes of uniform permeability, a method of preparation was adopted which had been suggested by Bigelow[9] and improved by Bartell and Carpenter.[10] 109 cc. of the collodion solution described above (2 gm. in 100 cc.) were poured on the surface of mercury in an enamelled iron tray about 27 × 33 cm., all bubbles were scraped over to the edge of the dish with a knife, and the dish was covered with a cardboard case about 32 × 49 × 50 cm. This retarded the evaporation of the solvents so that the time of drying was from 2 to 7 hours at about 20°C. At the end of this time water was poured directly on the collodion and the membrane was cut away from the sides of the tray and kept in water at least 1 day before use. These large membranes were cut into disks with a steel die 3.81 cm. in diameter. In each test-tube 3 disks were shaken with 25 cc. of gelatin solution, so that the apparent surface of the collodion was less than half of that used in the previous experiments. The dry weight of 3 disks varied from 0.073 to 0.089 gm., being determined separately for each membrane.

While the disks of each membrane were being shaken with the gelatin solutions, measurements of thickness and permeability were made with other disks of the same membrane. The thickness was determined by cutting a straight edge with scissors and observing the thickness of the edge with a micrometer microscope. Several measure-

[9] Bigelow, S. L., *J. Am. Chem. Soc.*, 1907, xxix, 1675.

[10] Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, xxvii, 101, 252.

ments were made with different freshly cut edges, and could be repro-
duced within 0.008 mm.

The permeability measurements were made at room temperature
by forcing water through the membrane in an apparatus similar to
that of Bartell and Carpenter.[10]   The area of membrane exposed to
pressure had a diameter of 2.1 cm., the highest pressure used was
about 75 cm. of water, and the rate of flow in a horizontal tube of
0.792 mm. diameter was determined with the aid of a stop-watch and
a millimeter scale.   The rate of flow was found to be quite closely pro-
portional to the pressure applied, which is in agreement with the ob-
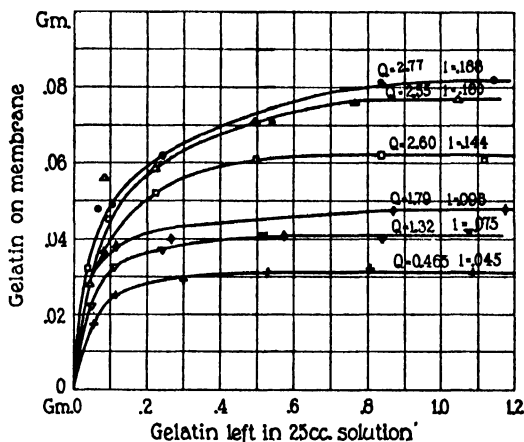
FIG. 8. Adsorption of gelatin by membranes of different permeabilities.

servations of Bigelow,[9] of Bartell and Carpenter,[10] and of Duclaux
and Errera.[11]

The results of adsorption experiments with gelatin and membranes
of different permeabilities are given in Fig. 8.   Each curve is marked
with the permeability of the membrane, Q, expressed in cu. mm. $\times$
10^{-4} of water flowing through an area of 1 sq. cm. of membrane per
second per mm. of water pressure, and with the thickness in mm., l,
of the membrane.   Each curve indicates a definite maximum amount
of adsorbed gelatin, being thus qualitatively in agreement with
Langmuir's equation.   In general the maximum amount of gelatin

[11] Duclaux, J., and Errera, J., *Rev. gén. colloïdes*, 1924, ii, 130.

adsorbed increased with the permeability and the thickness, the more permeable membranes being thicker.

It seemed of interest to compare the maximum amounts of adsorbed gelatin with the relative surface of the pores in the different membranes, as calculated from the permeability and thickness measurements by means of Poiseuille's law, which is

$$V = \frac{p \, \pi \, r^4 \, l}{8 \, L \, \eta}.$$

This law applies to the flow of a liquid through a capillary tube, and in the equation V = volume of liquid flowing in time t under pressure p through a tube of radius r and length L; η represents the coefficient of viscosity of the liquid. If the membrane is considered as a bundle of capillary tubes, the permeability as defined above is equal to $\dfrac{nV}{pt}$ where n is the number of tubes per sq. cm. of membrane area. Hence the permeability, Q, is given by the equation

$$Q = \frac{n \, \pi \, r^4}{8 \, L \, \eta}.$$

The length of the capillaries, L, is not known, but if it be assumed proportional to the thickness of the membrane, l, then Ql is proportional to r^4, or $r \propto \sqrt[4]{Ql}$, if the number of pores per sq. cm. of the membranes is taken as constant, which is in accordance with the conclusion of Bartell and Carpenter.[10] Hence the total pore surface of 1 sq. cm. of membrane, $2 \pi r L n$, is proportional to $l \, r$, or to $l \sqrt[4]{Ql}$. Fig. 9 shows a plot of the maximum amounts of adsorbed gelatin against the relative pore surface, or $l \sqrt[4]{Ql}$. The fact that the points lie nearly on a straight line seems to indicate that the maximum gelatin adsorbed is a linear function of the pore surface. This line cannot be extrapolated back to the axis of ordinates, for it was found that membranes of low pore surface adsorbed no gelatin. Hence the curve becomes discontinuous near its left end. Probably the discontinuity would lie at the point where the pores become too small to admit any gelatin at all.

Some measurements were made of the permeabilities of membranes, initially identical, after they had been treated with gelatin solutions of varying concentration. Fig. 10 shows that the square root of the
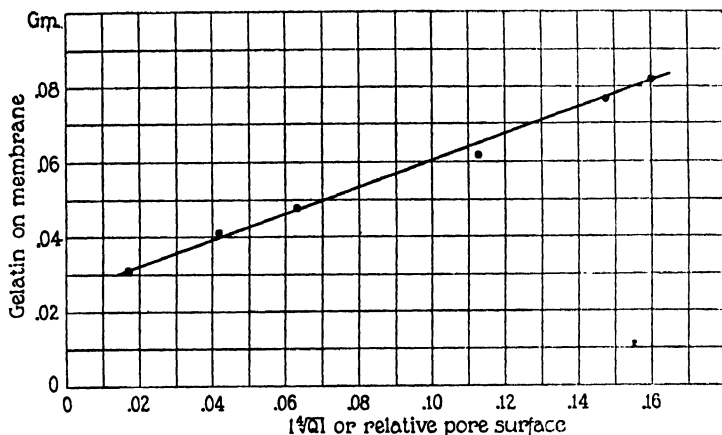
FIG. 9. Adsorption of gelatin as a function of relative pore surface as calculated from Poiseuille's law.
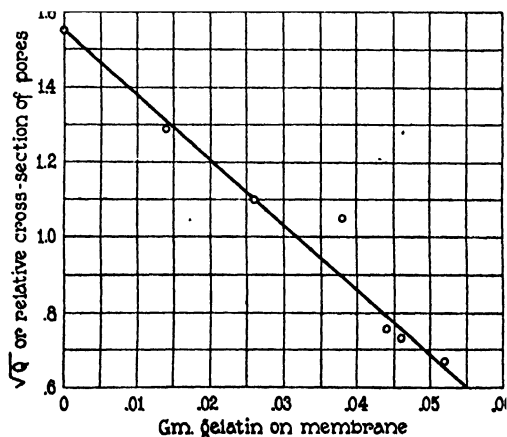
FIG. 10. Relative cross-section of pores of gelatin-coated membranes as a function of the amount of adherent gelatin.

permeability, Q, obtained as described above, was a linear function of the amount of gelatin on the membrane. Since in this case the thickness of the membrane, l, was constant, $\sqrt{Q}$ is proportional to r_1 if it is

assumed that the total number of pores remained the same. Hence the experiment shows that the cross-section of the pores left open after treatment with gelatin is a linear function of the amount of adherent gelatin. Thus Figs. 9 and 10 both confirm the idea that the adsorbed gelatin formed a layer on the surface of the pores. From the first and last points of Fig. 10 it can be calculated that the gelatin decreased the pore cross-section by 57 per cent or the radius by 34 per cent. On extrapolating the data of this experiment by means of Langmuir's equation and Fig. 10, it appears that the maximum amount of gelatin (0.068 gm.) which this membrane could have adsorbed would have decreased the pore cross-section by 76 per cent or the radius by 51 per cent.

SUMMARY.

1. Collodion membranes of high permeability were found to adsorb weighable amounts of gelatin and egg albumin from solution at 37°C.

2. The effect of protein concentration could be expressed fairly well by a hyperbolic equation proposed by Langmuir for the adsorption of gases by a plane surface, while the usual parabolic adsorption equation of Freundlich did not fit the results.

3. In comparing this effect with solutions of varying pH, it was found there was a decided maximum of adsorption in solutions of isoelectric protein. The effects of acids and salts on the amount of gelatin adsorbed were like those observed by Loeb on the viscosity of gelatin solutions, but opposite in direction. The effects of pH on the amount of adsorbed gelatin and on the fluidity of the gelatin solutions were nearly parallel.

4. Membranes made impermeable by long drying took up very little or no gelatin from solution.

5. In the case of membranes of varying permeability the maximum amount of adherent gelatin increased with the permeability and thickness of the membranes, and appeared to be, within limits, a linear function of the relative pore surface of the membranes as calculated from Poiseuille's law.

6. The film of gelatin greatly decreased the permeability of the membranes, as measured by the flow of water through them. The relative cross-section of the pore openings, as calculated from the

permeability measurements, was a linear function of the amount of adherent gelatin.   These results led to the conclusion that the gelatin formed a film inside the pores.

Many of the experiments described in this paper were carried out by Mr. C. E. Heinrichs.   The writer is indebted to Dr. J. H. Northrop for advice in connection with the work.

IS LIVING PROTOPLASM PERMEABLE TO IONS?*

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Cambridge, and The Rockefeller Institute for Medical Research.)

This question, answered in different ways by opposing schools, has become a center of controversy. Although each side has assembled an imposing array of facts, on every important point the evidence is conflicting and the interpretation doubtful. It is evident that the most satisfactory way of attacking the problem is to turn aside from the indirect methods which have resulted in contradictions and to aim rather at accurate measurements of actual penetration by direct analysis of the contents of the cell. We may hope that this procedure will clear up the existing confusion and prepare a sound basis for a theory of permeability.

The writer has been fortunate in finding an organism which allows such direct determinations to be made. It is a marine alga, *Valonia macrophysa*, Kütz.,[1] whose large, multinucleate cells sometimes contain as much as 10 cc. of sap, forming a central vacuole, which is surrounded by a delicate layer of protoplasm, outside of which is a cellulose wall.

To prepare the material for experiments clusters of cells were brought into the laboratory and the individual cells carefully separated. They were then allowed to stand for a day or more in order that any effect due to the handling might become manifest. Cells which showed any sign of injury were discarded.

Cells as nearly alike as possible in respect to size and shape were selected (the average volume was about 0.33 cc.). When smaller cells were attached to larger ones, care was taken to have as much similarity in the absorbing surfaces as could be obtained. Cells

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* Contributions from the Bermuda Biological Station for Research. No. 151.

[1] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225.

to which other organisms had attached themselves were rejected. In this way the conditions for absorption were made as nearly uniform as possible.

In the experiments here described the penetrating substance was H_2S. Cells placed in sea water containing various amounts of this gas were left until equilibrium was established between the H_2S inside and outside the cell. The concentration of total sulphide in the cell sap was then compared with that in the sea water.

The H_2S was prepared by the action of dilute c.p. HCl on FeS. The gas was passed through a column of cotton. In some experiments c.p. Na_2S and c.p. CaS were used to discover if the less pure H_2S obtained from the crude FeS differed in its action from that of the uncontaminated gas. No difference was found in the effects of the H_2S obtained from the two sources.

The H_2S was bubbled through sea water until the desired concentration was reached.[2,3] The cells were placed in bottles of 125 cc. capacity, filled to the brim with sea water containing H_2S: 12 cells were placed in each and the bottle immediately stoppered with a rubber stopper which was wired to hold it firmly in place. This eliminated any gas space, thereby helping to prevent oxidation of the sulphide during the experiment. When the pH value of the solution was varied the quantity of HCl or NaOH required to give the desired pH was placed in the bottle before pouring in the sea water. The stopper was inserted, the contents were then thoroughly mixed and a sample was taken for determination of the pH value. The bottles containing the cells were set aside until penetration was complete. This required about an hour for cells of this size, but in order to be sure that equilibrium was reached, the exposure was continued for

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[2] The concentrations were varied in different experiments but were usually not allowed to go above .06 M. It was found that the relation between total sulphide inside and outside was little affected by the total outside concentration so long as the pH value was kept constant.

[3] The sea water became cloudy with particles of sulphur, due to the oxidation of the H_2S by oxygen in the sea water. There is no objection to this since these particles do not affect the titrations and these cloudy solutions were used in the treatment of the cells. When, however, the pH of the solution was raised to 8 or higher by the addition of NaOH, as happened in some experiments, there resulted a perfectly clear solution.

2 or 2½ hours. A longer exposure was avoided because of the possibility of injury to the cell.

The analysis was made by treating the sample with a measured volume of .01 N iodine, and then titrating back the excess iodine with .01 N $Na_2S_2O_3$, using starch as indicator.

$$H_2S + I_2 = 2HI + S$$

$$I_2 + 2Na_2S_2O_3 = 2NaI + Na_2S_4O_6$$
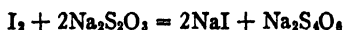
This measures the total sulphide ($H_2S + HS' + S''$).

At the close of the experiment the bottle containing the cells and sea water was shaken so as to mix the contents. The stopper was then removed and a specially constructed 3 cc. pipette was dipped into the sea water. This pipette was fashioned to reduce to a minimum the loss of H_2S in obtaining the sample for analysis. It consisted of 3 cc. bulb blown in a glass tube of 4 mm. bore, the bulb being drawn out at the lower end into a fairly large capillary and constricted at the upper end or graduation point. The tip of the pipette was ground with a file to a very sharp edge to facilitate pricking the cells in collecting sap. When the pipette was dipped in the sea water the finger was placed over the open end and the pipette taken out of the bottle, the stopper being replaced. The pipette was carefully wiped with a clean cloth and the water level allowed to fall to the graduation mark. The pipette was then dipped into a measured volume of iodine solution and its contents allowed to run out. Rinsing with distilled water followed. The flask was shaken and three drops of starch solution added. The excess iodine was then titrated with $Na_2S_2O_3$. The stopper was again taken from the bottle and the cells and sea water emptied into a finger-bowl. The cells were taken one by one from the sea water, a spot quickly wiped dry and this spot pricked by thrusting the sharp end of the pipette through the cell wall. Pressure applied to the cell by the fingers forced the sap up into the pipette. When filled slightly above the graduation mark it was carefully wiped, the water level allowed to fall to the graduation mark, and the contents of the pipette allowed to run into a second measured volume of iodine. Ordinarily it took 1 minute to collect 3 cc. of sap.

The question arises whether any H_2S is lost in obtaining the sap.

To decide this point, dead cells were placed in sea water containing H_2S and allowed to come to equilibrium. The samples of sap and of sea water were taken in the usual manner. Practically no difference was found between the sulphide content of the dead cell sap and that of the sea water, which indicates that little if any H_2S is lost in procuring the sap from the cells.

Since the sap has some reducing power it was desirable to determine its value. 3 cc. of sap from cells of ordinary size were treated with .01 N iodine and the excess titrated with .01 N $Na_2S_2O_3$. The reducing power ranged from .1 cc. to .2 cc. of .01 N iodine for 3 cc. of sap. A correction of .15 cc. .01 N iodine was therefore applied for each 3 cc. of sap (the titration usually consumed from 5 to 20 cc. of iodine solution, according to circumstances). The reducing action of the sea water was found to be negligible.

The pH values were determined colorimetrically; that of the external solution was taken as the figure obtained at the end of the exposure. The pH value of the sap of normal cells was found to average about 5.8. The pH value of the sap was not much affected during the time of the experiments by exposure to solutions with a pH range of 6.0 to 10.0. Outside of this pH range some influence of the external pH was observed, probably accompanied by injury.

The question arises whether the cells were injured by the treatment given them. It has been found that as soon as injury occurs the SO_4 of the sea water begins to penetrate. No penetration of SO_4 was observed during the time of the experiments unless the concentration of H_2S was very high and the pH value of the sea water fell below 6.0.[4]

At the end of each experiment all the cells that had been exposed to H_2S but which had not been pricked for the collection of sap were replaced in sea water and allowed to stand overnight. It was observed that after an exposure of 2 hours to H_2S there were often signs of injury the next day. It is not believed, however, that there was sufficient injury in any case to affect the results except possibly toward the end of the exposure in the highest concentrations at low pH values.

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[4] When the cell is killed the concentrations of SO_4 and total sulphide become the same in the sap as in the sea water.

The temperature varied somewhat from day to day (ranging from 20°C. to 22°C.) but there was very little variation during the time occupied by any experiment (on the average not more than one degree). The temperature coefficient is of the order characteristic of diffusion.
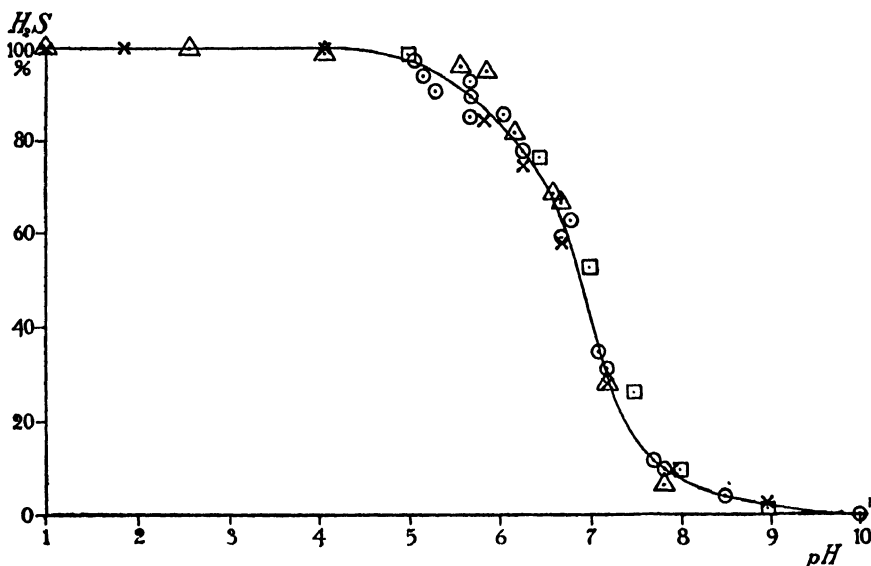
FIG. 1 demonstrates that the total sulphide in the sap corresponds with the undissociated H_2S in the external solution. The concentration of total sulphide ($H_2S + HS' + S''$) in the cell sap ($\circ$) is expressed as per cent of the total sulphide in the outside solution. The values for the concentration of undissociated H_2S in the sea water as calculated from the dissociation constant ($\square$) and as determined from the vapor tension ($\triangle$) and from the rate of evaporation ($\times$) at various pH values of the external solution are expressed as per cent of the corresponding values in the range pH 1 to 3 where all the H_2S is regarded as undissociated. Each point represents one determination.

The writer is indebted to Mr. William C. Cooper, Jr. for carrying out the experiments on penetration and on evaporation.

The results are shown in Fig. 1, in which the circles[5] denote the concentration of total sulphide ($H_2S + HS' + S''$) found in the sap of living cells at various pH values after equilibrium is reached between

[5] Each circle represents the value obtained by mixing the sap of 12 cells and titrating.

the total sulphide outside and that inside. The curve is drawn free hand through these points to give an approximate fit. The concentration of total sulphide inside the cell corresponds approximately to that of undissociated H_2S in the sea water outside as determined by the vapor tension ($\triangle$), the rate of evaporation ($\times$), and as calculated from the dissociation constant ($\square$).

The determinations of vapor tension were made by placing sea water containing H_2S in a bottle connected with a bottle of distilled water in such a way that a stream of gas could be made to circulate (bubbling through both solutions) by squeezing a rubber bulb. The whole formed a completely closed system, care being taken to make tight joints where the tubes passed through the stoppers, which were securely wired in place. The circulation of gas was continued until equilibrium was established between the H_2S in the sea water and that in the distilled water. The concentration of total sulphide in each was then determined by titration and the pH value was measured colorimetrically.

It was found that the lower the pH value of the sea water the higher was the relative total sulphide content of the distilled water as compared with that of the sea water; this was true down to pH 3 below which lowering of the pH value produced no increase in the relative concentration in distilled water. The H_2S was therefore regarded as undissociated at pH values below 3: the relative concentration of total sulphide in distilled water at these pH values was 116 per cent of that in sea water.[6] All the relative concentrations of total sulphide in distilled water were therefore multiplied by $\dfrac{100}{116}$ and the resulting figures were taken as expressing the per cent of undissociated H_2S present. Thus at pH 1 to 3 the per cent of undissociated H_2S is 100, at pH 5.6 it is 96.5 per cent and so on. These are approximate determinations: it is probable that if care had been taken to keep the pH value of the distilled water solution low enough

[6] It is to be expected that H_2S will be less soluble in sea water than in distilled water. *Cf.* Hildebrand, J. H., Solubility, American Chemical Society monograph series, New York, 1924, 140.

The ratio between the concentrations of total sulphide in the two solutions at any given pH of the sea water solution was not much affected by the variations in the total sulphide content of the sea water.

to prevent all dissociation of H_2S and if the formation of polysulphides by oxidation had been completely prevented there would be less irregularity. Each point on the curve represents one determination. The writer is indebted to Mr. M. J. Dorcas for these determinations.

In order to determine the rate of evaporation dishes of the same size (with straight sides and flat bottoms) were filled to the same height with solutions having the same concentration of total sulphide but brought to different pH values by the addition of acid or alkali. The rate of evaporation was ascertained by titration and was taken as the reciprocal of the time required to lose a given fraction of the total sulphide: this was ascertained by constructing time curves and taking the reciprocals after the loss of a fraction which was so small that the change in pH value due to the evaporation could be neglected (or a suitable correction made). The amount of excess base varied in these experiments but not sufficiently to alter markedly the dissociation curve as calculated by Becking's equation.[7]

It was found that the rate of evaporation increased with increasing acidity until the neighborhood of pH 3 was reached. As in the experiments on vapor tension the results indicated that at pH values below 3 the H_2S is not dissociated and in consequence the rate of evaporation at this point was taken as 100 per cent and all other rates expressed as per cent of that found from pH 1 to 3.

The per cent of undissociated H_2S as indicated on the curve by square symbols ($\square$) was calculated by means of the equation

$$H_2S = \frac{(H)\ (100 - H_2S)}{K}$$

in which the total sulphide is taken as 100 and K, the dissociation constant, is 0.91×10^{-7}. The equation neglects the dissociation of HS' into H^+ and S'' but the dissociation constant of this step is so small (probably about 10^{-15}) that it may be neglected. We may also neglect the effect of excess base for such calculations as are here required. These factors are taken into account in the equation given by Becking.[7]

$$H_2S = \frac{[(B) + (H)]\ (H)^2 - K_w(H)}{K_1(H) + 2K_1K_2}$$

[7] Cf. Becking, L. B., Proc. Soc. Exp. Biol. and Med., 1924–25, xxii, 127.

in which K_1 is the dissociation constant for $H_2S = H^+ + HS'$, K_2 the dissociation constant for $HS' = H^+ + S''$, $K_w = (H)(OH) = 10^{-14}$, and B (excess base in relation to H_2S) is defined by the equation $(B) + (H) = (OH) + (HS) + 2(S)$. The values given by this equation do not differ sufficiently from those obtained by the simple equation employed above to make any marked difference in the curve.

Fig. 1 shows that the concentration of total sulphide inside the cell agrees very well with that of undissociated H_2S in the sea water outside, indicating that only undissociated H_2S can penetrate and that it does not become much dissociated after entering the cell.

This may be illustrated by a simple example. Let us consider an experiment in which the pH value of the outside solution at the start is the same as that of the sap, the excess base inside and outside being equal and the volume of sea water being very large as compared with that of the cell. Let us assume that only undissociated H_2S enters the cell and that it does not change its degree of dissociation after entering the cell (since the inside and outside pH values are the same). If the solubility of H_2S is the same in sap and sea water the concentration of H_2S inside and outside will be the same: this will also be true of the ions and consequently of the total sulphide (ions plus undissociated molecules). At the usual pH value of the sap (about 5.8) H_2S is about 5.4 per cent dissociated, so that if we call the total sulphide in the sea water 100 the concentration of undissociated H_2S in both sap and sea water is 94.6. Let us now raise the pH value of the outside solution to pH 7.05 at which the concentration of undissociated H_2S in the sea water is only 50: undissociated H_2S will move out of the cell until its concentration in the sap becomes the same as that in the sea water (50). The concentration outside will show practically no increase owing to the relatively large volume of sea water. We find that the pH of the sap does not rise noticeably, at least not during the time of the experiment, and we should have in the sap a concentration of 50 undissociated H_2S and $50\left(\dfrac{5.4}{94.6}\right) = 2.9$ ionized molecules, so that the total sulphide in the sap is 52.9, which would correspond fairly well with the concentration of undissociated H_2S outside (50). Proceeding in this way to higher pH values, keeping the total sulphide outside

constant at 100 and the pH value inside constant at 5.8, we should obtain a result like that given in the figure, but the curve showing the concentration of total sulphide in the sap would be $\frac{5.4}{94.6}$ = 5.7 per cent higher than that showing the concentration of undissociated H_2S in the sea water.

Instead of raising the outside pH value from the starting point (5.8) we might lower it, for example to pH 5.5, at which the concentration of undissociated H_2S (if we keep the total sulphide outside constant at 100) would be 97.2. If the inside pH value remained constant the inside concentration of total sulphide would be 97.2 + (97.2 × $\frac{5.4}{94.6}$) = 102.8; if the outside pH value were low enough (pH 3 or lower) the concentration of total sulphide outside would be 100 and that inside would be 100 + $\frac{5.4}{94.6}$ = 105.7.

We might expect the concentration inside to be even higher because H_2S might be more soluble in sap than in sea water since the sea water contains Mg, Ca, and SO_4 which would tend to make H_2S more soluble in the sap.[8] In order to test this idea an artificial sap[9] was made by dissolving NaCl and KCl in water in the proportions in which they occur in sap. A current of air was passed in succession through sea water containing H_2S and through the artificial sap, the whole forming a closed system as in the vapor tension experiments. It was found that at equilibrium the artificial sap contained about 13 per cent more total sulphide than the sea water.

We should therefore expect to find an excess of undissociated H_2S in the sap as compared with the sea water, but this excess would be lessened if the cell produced enough CO_2 to make its concentration higher in the sap than in sea water (photosynthesis would act in the opposite direction); this would tend to diminish the solubility of H_2S in the sap.[8] Such a diminution was actually observed when CO_2 was added to artificial sap.[9] The concentration of CO_2 in the sap might be subject to some fluctuation: it is usually less than in sea water.

The presence of organic matter in the sap might affect the solubility

[8] *Cf.* Hildebrand, J. H., Solubility, American Chemical Society monograph series, New York, 1924, 140.
[9] This was made by mixing 86.24 cc. of KCl .6 M with 15.08 cc. of NaCl .6 M.

of H₂S in either direction but the amount of organic matter is very small.[10]

As a matter of fact we do not find an excess of total sulphide in the sap as compared with the undissociated H₂S of the sea water. The reason for this is not wholly clear but at all the pH values below 5.8 it might be due in part to the fact that the penetrating H₂S lowers the pH of the sap (as is actually observed) which would tend to diminish the excess of total sulphide inside.[11]

Our present problem concerns itself with the entrance of ions into the protoplasm rather than with the extent to which substances accumulate in the cell. We therefore wish to know whether there is a general correspondence between the undissociated H₂S in the sap and in the sea water. If such a correspondence exists we shall get similar curves when we plot the total sulphide in the sap (expressed as per cent of total sulphide outside) and at the same time plot the per cent of undissociated H₂S in the outside solution. This has been done in Fig. 1 and the result is very striking. The concentration of undissociated H₂S inside agrees so closely with that outside that we can hardly escape the conclusion that it is only the undissociated molecules which penetrate.[12] If the protoplasm is practically impermeable

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[10] The organic matter amounts to 1.433 parts per thousand.

[11] This effect of the penetrating H₂S would depend on the buffer action of the sap which is apparently small. If the exposure is prolonged at pH values below 5 injury occurs as shown by the entrance of SO₄.

[12] If we suppose that H⁺ ions can diffuse in and out of the cell (an improbable assumption in view of the fact that the inside pH remains about 5.8, while that of sea water is about 8.2) and if indiffusible ions are present and Donnan equilibrium is set up with HS′ ions passing in freely, but not undissociated H₂S, we should expect the relation (ignoring the formation of S″ ions).

$$\frac{H^+ \, inside}{H^+ \, outside} = \frac{HS' \, outside}{HS' \, inside}.$$

For example when the inside pH value is 5.8 and the outside is 5.8 $\frac{H^+ \, inside}{H^+ \, outside} = 1$ and we should expect to find $\frac{HS' \, outside}{HS' \, inside} = 1$. As a matter of fact we find the value to be close to 1. When $\frac{H^+ \, inside}{H^+ \, outside} = 10$ we find $\frac{HS' \, outside}{HS' \, inside} = $ about 10 and so on.

If, however, it is HS′ and not undissociated H₂S which penetrates we should expect to find the rate of penetration highest when the outside concentration of HS′ is highest; i.e., at high pH values. But this is not necessarily the case for

tu ions but allows undissociated molecules to enter freely we should expect precisely the result we have found.[13]

In this connection it may be noted that the work of Beerman[14] on H_2S and that of Loeb, Harvey, Crozier, Haas, Jacobs, Brooks, Smith, Clowes, and others[15] (on various weak acids) indicates that undissociated molecules penetrate although the methods employed do not enable us to decide positively whether ions enter or not. Those who have concluded that ions cannot penetrate have done so on purely theoretical grounds or as the result of indirect evidence.

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when the outside concentration of HS' is highest the concentration which it finally reaches in the sap is lowest and hence the rate of penetration might be proportionally low. Since we actually find that the rate of penetration is highest when the outside concentration of undissociated H_2S is highest; i.e., at low pH values, it seems probable that only undissociated H_2S can penetrate. Moreover, certain unpublished results make it difficult to assume that ions can penetrate while undissociated molecules are unable to do so.

In general we cannot assume that Donnan equilibrium is the primary factor since in that case the relation of K^+ and Na^+ inside to K^+ and Na^+ outside should be the same as that of H^+ inside to H^+ outside. This is approximately true for K^+ under normal conditions, but not for Na^+ which has a much higher concentration in the sea water than in the sap. If Donnan equilibrium prevailed the concentration of Cl' ions should be about a hundred times as great outside as inside but it is actually somewhat greater inside. If any of these ions are not diffusible, as such, these remarks would not apply to them.

[13] When the pH was varied but the concentration of undissociated H_2S in the outside solution was kept constant by adjusting the concentration of total sulphide it was found that the concentration of total sulphide in the sap at equilibrium remained unaltered despite changes in the pH value of the outside solution.

[14] Beerman, H., J. Exp. Zool., 1924–25, xli, 33.

[15] Loeb, J., Biochem. Z., 1909, xv, 254; 1910, xxiii, 93. Arch. ges. Physiol., 1897–98, lxix, 1; 1898, lxxi, 457. Artificial parthenogenesis and fertilization, Chicago, 1913, 143. J. Gen. Physiol., 1922–23, v, 231. Harvey, E. N., Internat. Z. physik.-chem. Biol., 1914, i, 463. Carnegie Institution of Washington, Pub. No. 212. 1915. Crozier, W. J., J. Gen. Physiol., 1922–23, v, 65, with references to earlier papers. Haas, A. R. C., J. Biol. Chem., 1916, xxvii, 225. Jacobs, M. H., Am. J. Physiol., 1920, li, 321; liii, 457. Biol. Bull., 1922, xlii, 14. Brooks, M. M., Pub. Health Rep., U. S. P. H., 1923, xxxviii, 1449, 1470. Smith, H. W., and Clowes, G. H. A., Am. J. Physiol., 1924, lxviii, 183. Smith, H. W., Am. J. Physiol., 1925, lxxii, 347.

If it should turn out to be generally true that ions are unable to penetrate how shall we regard the evidence for the contrary view? This evidence rests chiefly on experiments with plasmolysis and electrical conductivity. It is found that many cells recover after plasmolysis when left in the plasmolyzing salt solutions (provided they are not too concentrated). Since these salts are largely ionized this may be regarded as evidence of permeability to ions. It is, however, quite possible that in these experiments the cells are permeable to ions only because they are abnormal. It is well known that plasmolysis produces injury and that injury is accompanied by changes in permeability. It is also possible that the cell may subsequently recover from such injury and appear to be normal: in this case the permeability to ions would be only a temporary one. Injury might affect only a portion of the cell surface, possibly numerous small areas. Experiments on large multinucleate cells (*Valonia, Nitella, Caulerpa, Bryopsis*) have convinced the writer that a portion of the cell surface may be greatly altered while the remainder remains in normal condition for a long time afterward.

If recovery from plasmolysis in salt solutions depends on alterations of permeability we should expect the rate of recovery from plasmolysis to correspond somewhat with the amount of alteration. If the alteration goes too far the cell may become so permeable that no recovery is possible, but up to a certain point increase in permeability would increase the rate of recovery from plasmolysis if exosmosis were not greater than endosmosis. From this standpoint we might expect the recovery in NaCl to be more rapid than in a balanced solution of NaCl + CaCl$_2$ (or in sea water) since alterations in permeability would be more rapid in NaCl. We find that recovery is more rapid in NaCl than in balanced solutions. When recovery occurs in balanced solutions it is possible that it is also due to alterations in permeability, since it is well known that hypertonic balanced solutions may cause injury.

If ions are unable to penetrate normal protoplasm how are we to regard the experiments which indicate that marine plants bathed in sea water allow ions to enter the protoplasm and thus conduct the electric current under conditions which seem to ensure that the cells are in a normal state?

It is possible that if the cell normally opposes a high resistance to the passage of ions this resistance may be overcome under electric stress so that ions may be forced through the surface of the protoplasm, although they would not enter if the electric potential were absent. In this case the measurement of electrical conductivity would reveal changes in resistance to the passage of ions brought about by various conditions, but the passage of the electric current would not mean that ions could penetrate to an appreciable extent in the absence of an applied potential. From this standpoint we may say that the general conclusions derived from electrical experiments would not be changed except that the normal cell would not be regarded as permeable to ions. The measurement of changes in resistance to the passage of ions brought about by abnormal conditions and the conclusions drawn from these measurements would still be valid.

It is also possible that the measurements of conductivity do not indicate the passage of ions through the protoplasm, as has been supposed. If the cell acts as a condenser an alternating current may seem to pass without actual transfer of ions through the protoplasm as indicated, for example, by the recent experiments of McClendon.[16] Experiments to test this were carried out in the writer's laboratory by Mr. M. J. Dorcas several months before the appearance of McClendon's article and have been continued by Mr. L. R. Blinks. If this turns out to be true the increase in conductivity (as measured by the alternating current) which occurs when a cell is injured may be regarded as analogous to the change by which a condenser becomes a conductor. If the cell surface is covered with a non-conducting substance injury might result in the alteration of this substance in certain places, so that the conductivity would increase. If this view should turn out to be correct we should still regard the measurements of the electrical conductivity of living tissues by means of the alternating current as of great value in detecting changes of permeability.

The principle that only the undissociated molecules can enter the protoplasm has far reaching implications. Among those we may mention the general question of equilibrium relations. In the case of a weak acid it is evident from what has been said that at equilibrium

[16] McClendon, J. F., *Science*, 1924, lx, 204.

when the internal pH value is greater than the external the total concentration of the weak acid (ionized plus unionized) will be greater inside than outside, and the reverse will be true when the internal pH value is less than the external. In the case of a weak base these relations will be reversed. It might be thought that a base penetrating into acid cell sap would soon raise the inside pH value to that found outside but this may be delayed by the buffer action of the sap or by the continuous production of acid (*e.g.* carbonic) within the cell. In this way ammonia might continue to accumulate in the sap, being changed into ions (or changed in some other way) as it enters and thus rendered incapable of escape, so that its concentration would become much greater inside the cell than outside. This apparently happens in the case of *Valonia*. We should not expect acids from the outside solution to accumulate in the sap, unless the outside pH value is lower than that in the sap; under such conditions the cell soon dies. Moreover, we should not expect to find a continuous production of alkali in the cell such as would be needed to ensure a great accumulation of acid. But if organic acids are produced inside the cell they might not be able to escape rapidly and hence the sap might remain acid. As a matter of fact we find the concentration of H^+ ions in the sap to be as a rule about 100 times as great as in the sea water.

We may extend this hypothesis to include not only weak acids and bases but all other substances, organic or inorganic, which are able to change with changes in the concentration of H^+ and OH' ions. Such changes (including tautomerism, formation of complex salts, hydration, hydrolysis, etc.) may affect substances to a greater extent than is at present suspected and may explain the accumulation in the cell sap of substances whose behavior now seems unaccountable. (When we say that a substance accumulates we mean that it becomes more concentrated in the sap than in the sea water.)

Substances which accumulate in this way should come out when the external pH is made equal to the internal, provided the cell has not in the meantime produced additional acid or base. The writer's experiments indicate that when the outside pH is made equal to that of the sap injury occurs and accumulated substances begin to come out after a few hours.

It is to be expected that accumulation could also occur as the result of combination with organic constituents of the cell. This has recently been emphasized by Miss Irwin as the result of experiments on dyes.[17]

If ions are unable to penetrate (except possibly to a slight extent) how shall we account for the presence of KCl and NaCl inside the cell? The experiments so far carried out suggest that these substances enter so slowly that their penetration may be accounted for by supposing that only the undissociated molecules enter or that the ions enter with extreme slowness. These experiments will be discussed in a subsequent paper.

It is not to be expected that all undissociated molecules can enter the protoplasm with the same readiness and in many cases it appears as though they cannot penetrate into the sap unless they can combine chemically with some constituent of the protoplasm.

If we adopt the hypothesis that ions enter normal protoplasm very slowly or not at all it is evident that injury and death are accompanied by increased permeability to ions. There is good evidence that this is the case.[18]

The results described above might be thought to be explainable on the hypothesis that the protoplasm is permeable to kations but not to anions, or *vice versa*, as appears to be the case with some membranes.[19] Unless ions of like sign were exchanged no ion could penetrate unless accompanied by one of opposite charge, and in case such exchange were very slow penetration would be practically confined to undissociated molecules. Under ordinary conditions there seems to be little or no exchange of ions: otherwise it is difficult to see how the differences between sap and sea water in respect to ionic concentration can be maintained. These differences are found in anions as well as in kations.[1]

Numerous questions suggested by what is said above must be deferred to subsequent papers for discussion. The object of the

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[17] Irwin, M., *J. Gen. Physiol.*, 1925-26, viii, 147.

[18] *Cf.* Osterhout, W. J. V., Injury, recovery, and death, Monographs on experimental biology, Philadelphia and London, 1922.

[19] *Cf.* Michaelis, L., *J. Gen. Physiol.*, 1925-26, viii, 33.

writer is merely to present certain facts with a tentative outline of some theoretical matters which will be taken up later as occasion offers.

SUMMARY.

The experiments indicate that under normal conditions little or no H_2S enters the cell sap of *Valonia macrophysa* except as undissociated molecules.

ACCUMULATION OF BRILLIANT CRESYL BLUE IN THE SAP OF LIVING CELLS OF NITELLA IN THE PRESENCE OF NH₃.

By MARIAN IRWIN.*

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I.

INTRODUCTION.

It has been shown by several investigators[1] that when the pH value of the external dye solutions is increased, the rate of accumulation of a basic dye in the cell sap is accelerated. Two different explanations for this have been given. Some have accounted for it on the basis that the basic dye enters the cell in the form of a dye hydrate and combines with the substances in the cell; and that, therefore, the increase in the rate of accumulation of the dye is due to the increase in the concentration of the dye hydrate. Others[2] have assumed that the increase in the rate of accumulation of the dye is due primarily to the increase in the concentration of the combining substances in the cell caused by the increase in the pH value of the cell contents. From these standpoints it is of decided interest to see what will happen if we increase the pH value of the cell sap, while keeping that of the external solution constant. An investigation of this sort was made by McCutcheon and Lucke,[3] but from their

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* This work was done in part while the writer held a fellowship in the biological sciences, National Research Council, Washington, D. C.

[1] Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

[2] Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. For other recent papers on the influence of pH on vital staining see Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354. Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223, 727.

[3] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

experiments it is not possible to draw a definite conclusion.[4]   They placed living cells of *Nitella*[5] in dye solutions at pH 8, containing in one case NH_4OH and in another case NaOH, and found that the dye accumulated in the sap less rapidly in the former than in the latter. Since the pH value of the cell sap increased in NH_4OH solution (without dye) while it did not change from the normal in NaOH solution (without dye) they concluded that the decrease in the rate of accumulation of the dye in the sap was due to the increase in the pH value of the sap, and that therefore the dye entered in the form of a dye hydrate, DOH, and combined with weak acids in the sap.   This conclusion, however, does not seem to be entirely justified for the following reason.   The change in the pH value of the sap is merely an indication that NH_3 (for convenience the term NH_3 will be used in this paper to represent aqueous NH_3 which includes undissociated ammonium compounds and NH_4 ions), has combined with substances in the sap, thus decreasing their power to combine with the dye when it enters.   In other words, there is a competition between the dye and NH_3 for the substances in the sap and it is quite possible that the competition may exist without noticeable increase in the pH value of the sap.   If there is sufficient buffer action, a considerable amount of NH_3 might accumulate without raising the pH value, but this would diminish the rate of .accumulation of the dye since the NH_3 would compete with the dye for the substances in the cell.   The presence of such a competition does not necessarily mean that the dye enters the cell in the form of DOH, and that the combining substances are weak acids.   In case the dye salt, DCl, unites with the salts of proteins or of weak acids, the rate of accumulation of the dye will be decreased when NH_3 is present in the cell, provided the affinity of NH_3 for the substances in question is greater than the affinity of the dye for the same substances.   The reaction might be of the ordinary type where NH_3 combines with proteins or weak acids to form salts which are ionized and which can combine with the dye or NH_3 might combine with a salt to form a compound which

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[4] *Cf.* Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[5] The same results were obtained by them with *Gonionemus* and starfish eggs. See Foot-note 3.

cannot combine with the dye, similar to the compound[6] formed by the union of copper hydrate with sodium tartrate, with which sodium hydroxide cannot react.

The possibility must also be borne in mind that NH_3 in the external dye solution may hinder the dye from entering the cell. McCutcheon and Lucke concluded, on the ground of experiments with the effect of NH_3 on the partition of dye between oil of sweet almonds and water, that the presence of NH_3 had no effect upon the taking up of the dye by the oil, but this may not necessarily prove to be the case with living cells.

In order to determine the cause of the decrease in the rate[7] of accumulation of dye in the presence of NH_3 it is desirable to carry out experiments which will show the rate of accumulation of the dye in the sap; (1) when the pH values of the sap are the same, while the concentrations of NH_3 in the sap are varied, which will show if there is a competition between NH_3 and the dye in the sap without a change in the pH value of the sap; (2) when the pH values of the sap and the concentrations of NH_3 in the sap are the same while the concentrations of NH_3 in the external solutions are varied, which will show if the presence of NH_3 in the dye solution can hinder the penetration of the dye into the cell; and (3) when the pH values of the sap are varied, while the concentrations of NH_3 in the sap remain practically constant, which will show if an increase in the pH value of the sap alone can bring about a decrease in the rate of accumulation of the dye.

[6] Norris, J. F., The principles of organic chemistry, New York, 1912, 272.

[7] It is evident that the competition of NH_3 would affect both the rate of accumulation of the dye in the sap and the concentration of the dye in the sap at equilibrium. In this paper the rate alone is studied since the cells die before the final equilibrium is attained. The rates are taken as near the beginning as possible and represent the concentrations of the dye in the sap at a given time in all cases. It is assumed that such rates would be approximately proportional to the concentrations of dye in the sap at equilibrium, if the reaction were not complicated by secondary processes as described in the writer's previous paper (see Foot-note 4).

<div align="center">II.</div>

Accumulation of Brilliant Cresyl Blue in the Sap When Living Cells Are Placed in a Solution of Dye Containing NH_4Cl.

In order to see how much decrease occurs in the rate of accumulation of the dye in the sap when the cells are placed in a solution of dye containing NH_4Cl, cells were divided into three lots. The
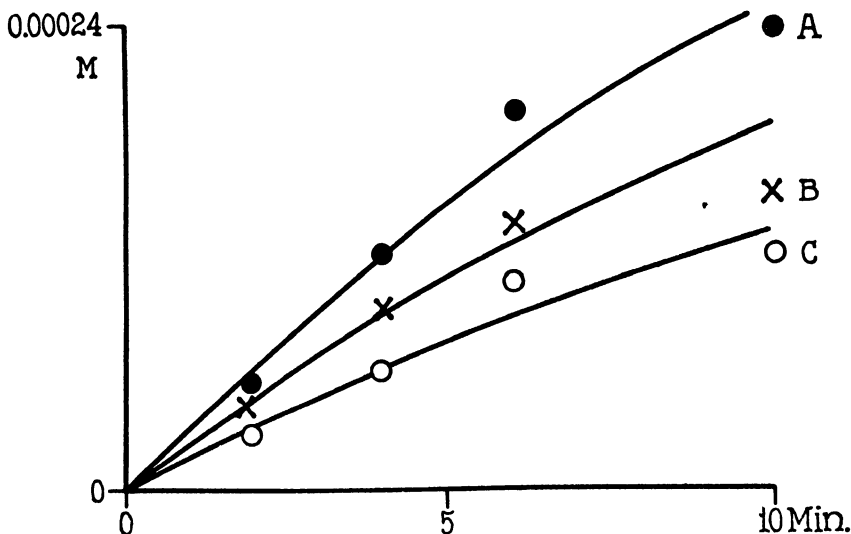
FIG. 1. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella*. The ordinates represent the concentrations of dye in the sap, and the abscissæ represent time. Curve A shows the rate of accumulation in 0.00014 M dye solution at pH 6.9; Curve B shows the rate in 0.00014 M dye solution at pH 6.9 containing 0 005 M NH_4Cl; Curve C shows the rate in 0.00014 M dye solution at pH 6.7. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

first lot was placed in 0.00014 M dye solution at pH 6.9, the second lot in the same concentration of dye solution at the same pH containing 0.005 M NH_4Cl, and the third lot in the same concentration of dye solution at pH 6.7. The concentration of the solutions was kept constant. All solutions were made up with $\dfrac{M}{150}$ phosphate buffer mixtures.[8] The experiments were made at 25 ± 0.5°C.

[8] The writer wishes to thank Mr. E. S. Harris for determining the pH values of the solutions by means of the hydrogen electrode.

At definite intervals, a few cells were removed, wiped, and the end of each cell was cut and the sap gently squeezed out on a glass slide. The sap was then drawn up into a capillary tube, and the color of the
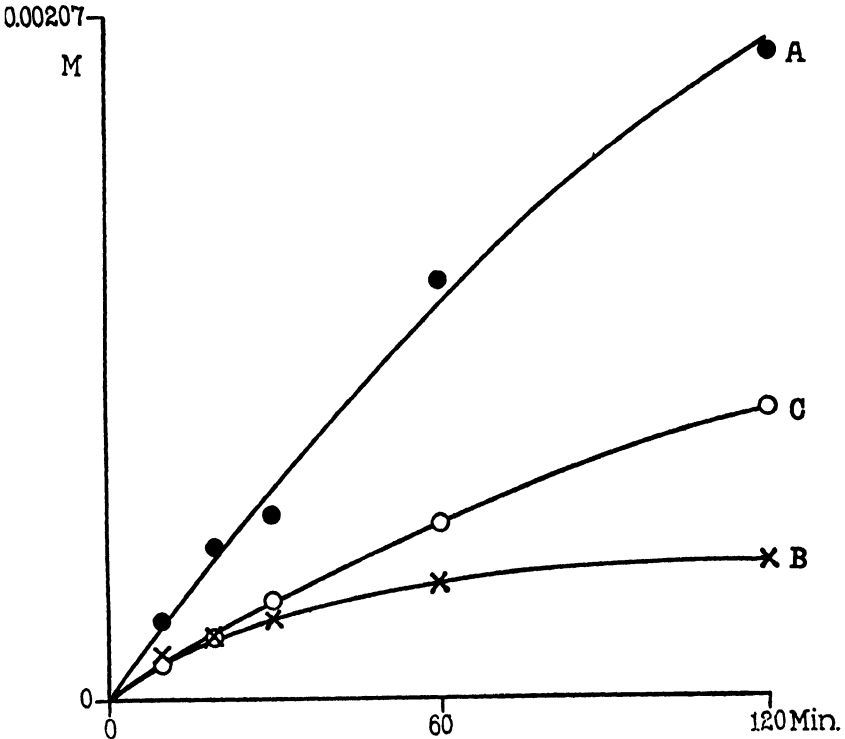
FIG. 2. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella*. The ordinates represent the concentrations of dye in the sap, and the abscissæ represent time. Curve A shows the rate of accumulation in 0.00014 M dye solution at pH 6.9; Curve B shows the rate in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH₄Cl; Curve C shows the rate in 0.00014 M dye solution at pH 6.7. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

tube was matched[9] with that of a tube of the same diameter containing a known concentration of the dye. The results are shown in Figs. 1 and 2.

On comparing the rate of accumulation of the dye in these three

[9] For details of technique see the writer's previous paper, *J. Gen. Physiol.*, 1925–26, viii, 147. In all the figures the curves are drawn free-hand through the points to give an approximate fit.

different dye solutions, it is found that in the first lot the rate is the highest from the start, while in the second lot the rate is higher at the start than in the third, but it becomes lower after about 25 minutes, as shown in Figs. 1 and 2, Curves A, B, and C.   These curves indicate clearly that when the cells are placed in a solution of dye containing NH₄Cl, the rate of accumulation of the dye in the sap falls off from the start and this decrease becomes greater as the time goes on.

III.

Change in the pH Values of the Cell Sap.

In order to carry out such experiments as are discussed in the introduction it is first of all necessary to obtain time curves showing the changes in the pH values of the cell sap when living cells are placed in solutions with[10] and without NH_3.

The pH of the sap was determined by the colorimetric method as follows: A capillary tube was filled for 1 inch with the sap; another capillary tube of the same diameter was filled for $\frac{1}{10}$ of an inch with about 0.005 per cent brom-cresol purple.   The contents of the two tubes were then mixed on a glass slide and the entire amount of mixed sap and indicator was drawn up into a third capillary tube the color of which was carefully matched with that of a fourth tube having the same diameter as the third and filled with a standard phosphate buffer solution of known pH value containing the same concentration of the indicator as the third tube.   The sap contains about 0.1 M halides so that the salt error should be corrected to obtain absolute values, but since the importance of these experiments lies in the relative values, this correction was omitted.

The solutions of NH₄Cl were made up in $\frac{M}{150}$ phosphate buffer mixtures at pH 6.9, and the concentration was kept constant throughout the experiment.   The experiments were made at 25 ± 0.5°C.

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[10] Hoagland, Davis, McCutcheon, Lucke, and the writer have found that the increase in the pH value of the cell sap takes place when cells are placed in a solution containing NH_3.   See Foot-notes 3 and 4, and also, Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

When the cells were placed in 0.005 M NH₄Cl solution at pH 6.9 and the change in the pH value of the sap was followed at definite intervals until death took place, it was found that after 5 minutes the pH value of the sap began to increase and in about half an hour it changed from pH 5.6 (normal) to pH 5.94 beyond which there was very little change in the pH value as shown in Fig. 3, Curve A, until almost all the cells were dead (within 6 hours after they were placed in the solution).
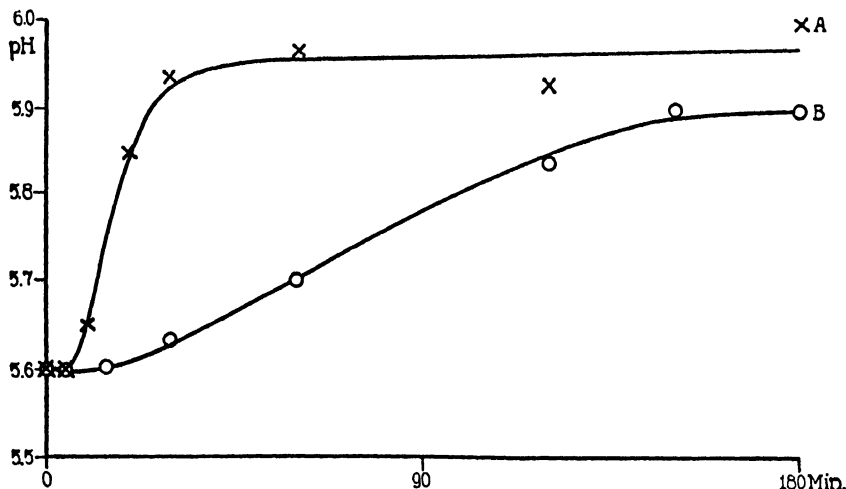
FIG. 3. Time curves showing the change in the pH value of the sap of living cells of *Nitella*. The ordinates represent the pH values of the sap, and the abscissæ represent time. Curve A shows the changes in the pH value when the cells are placed in 0.005 M NH₄Cl at pH 6.9. Curve B shows the changes in the pH values when the cells are placed in boric acid and sodium hydrate mixtures at pH 10.1. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

When living cells were placed in a buffer solution at pH 10.1 $\left(\dfrac{M}{40}\right.$ boric acid + NaOH), and the pH value of the sap was determined at intervals, it was found that the pH value began to change in about 20 minutes and in 2½ hours increased from 5.6 to 5.9, after which there was a very little change as shown in Fig. 3, Curve B, until almost all the cells were dead (in about 5 hours).

On comparing Curves A and B in Fig. 3, it is found that the pH value of the sap changes more rapidly, and the flattening of the curve is reached more quickly in the case of the cells placed in 0.005 M NH$_4$Cl solution at pH 6.9 $\left(\dfrac{M}{150} \text{ phosphates}\right)$ than in the case of the cells placed in pH 10.1 $\left(\dfrac{M}{40} \text{ boric acid } + \text{ NaOH}\right)$ without NH$_4$Cl.

If we base our notion of injury to the cells on the rapidity of death in the solution, then there is a very little difference between the cells placed in these two solutions, in that the cells in 0.005 M NH$_4$Cl solution at pH 6.9 die in about 6 hours while the cells placed in pH 10.1 solution without NH$_4$Cl die in about 5 hours. But if we base the criterion of injury[11] on the power of recovery there is a considerable difference as shown by the following experiment. When the cells that had been placed in 0.005 M NH$_4$Cl solution for 1 hour and other cells that had been in pH 10.1 buffer solution for 2½ hours were removed from the solutions, wiped, and placed in distilled water (in which the cells normally live for days), almost all the former cells were found living after 24 hours, while the latter were almost all dead in 2 hours. In all probability cells become more or less injured when the pH value of the sap is appreciably changed but the injury to the cells under these conditions in NH$_4$Cl solution is much less and the recovery is more apt to occur than when the cells are placed in NaOH plus boric acid buffer solution. This injury increases and the cells die if left in these solutions for several hours. It is not possible to change the pH values of the cell sap in solutions containing NH$_3$ or NaOH in such a way that one can definitely say that the cells are not injured at the time the pH value of the cell sap is altered. The conditions described above are the most favorable which the writer has been able to find.

[11] If we base the criterion of injury in the appearance of masses of chlorophyll in the sap, the observation is not accurate, though in general we may state that there are greater masses of chlorophyll in the sap of an injured cell than in the sap of a normal cell.

<div style="text-align:center">IV.</div>

*Accumulation of NH₃ in the Sap When Living Cells Are Placed in
NH₄Cl Solution.*

Since the experiments in Section III show that when the cells
are placed in NH₄Cl solution the pH value of the sap increases pro-
gressively until an apparent equilibrium is established, it is desirable
to see what type of curve is followed in the accumulation of NH₃ in
the sap.

Cells were placed in 0.005 M NH₄Cl solution at pH 6.9 $\left(\frac{M}{150}\right.$ phos-
phates$\left.\right)$ at 25 ± 0.5°C. The concentration of the solution was
kept constant. The concentration of NH₃ in the sap was deter-
mined by means of Nessler's reagent in the following manner. A
few cells were removed from the solution and wiped with a wet
cloth (free from NH₃). The ends of the cells were cut and the sap
was gently squeezed out on a glass slide. Then the sap was drawn
up into a capillary tube (about 10 inches in length), until it filled
the tube for the distance of 1 inch. The sap was then blown into
the Nessler tube containing 50 cc. of distilled water and 1 cc. of the
Nessler reagent. The solution was then carefully shaken. A
standard solution was made by taking the same amount (as in the
case of the sap) of a known concentration of NH₄Cl solution and
putting this into a Nessler tube containing 50 cc. of distilled water
and 1 cc. of the Nessler reagent in the same manner as in the case
of the sap. The colors of the two tubes were then compared by
looking into the solutions from the top of the tubes. Since the
color of the solution deepened on standing in both cases, it was
necessary to make determinations at a definite time after the solu-
tions were made up. For the present purpose the color was matched
immediately.

The sap is not a clear liquid like the standard solution but it con-
tains a viscous substance, which rises to the top of the tube and
makes the readings difficult so that an accurate determination of an
absolute value of NH₃ is not possible but this did not interfere with
the present experiments since we needed only such relative values
as could be obtained by this method.

The distilled water did not contain a measurable amount of NH_3 so that this was considered to be at a zero concentration for the sake of comparison since this distilled water was used for the Nessler test.

Tests showed that NH_3 adhering to the surface was not sufficient
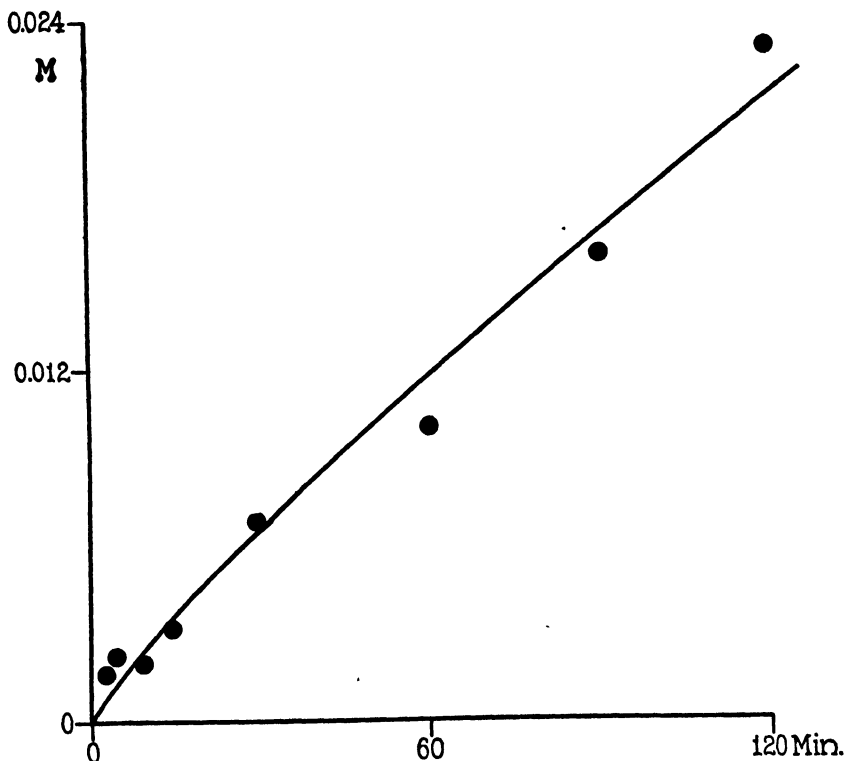
FIG. 4. Curve showing the rate of accumulation of NH_3 in the sap when living cells of *Nitella* are placed in 0.005 M NH_4Cl at pH 6.9. The ordinates represent the concentrations of NH_3 in the sap and the abscissæ represent time. Each point on the curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

to bring about noticeable errors. When the cells were dipped for a second in 0.005 M NH_4Cl and then wiped, and the sap was examined for NH_3 as described above, it was found that the sap gave no test for NH_3.

When living cells were placed in 0.005 M NH_4Cl at pH 6.9 and a few were removed at definite intervals for the determination of NH_3

in the sap, it was found that the accumulation of NH_3 took place gradually in the sap without reaching an equilibrium before the cells died (in about 6 hours). See Fig. 4.

When the relation between the concentration of NH_3 in the sap and the extent of the change in the pH value of the sap is considered,
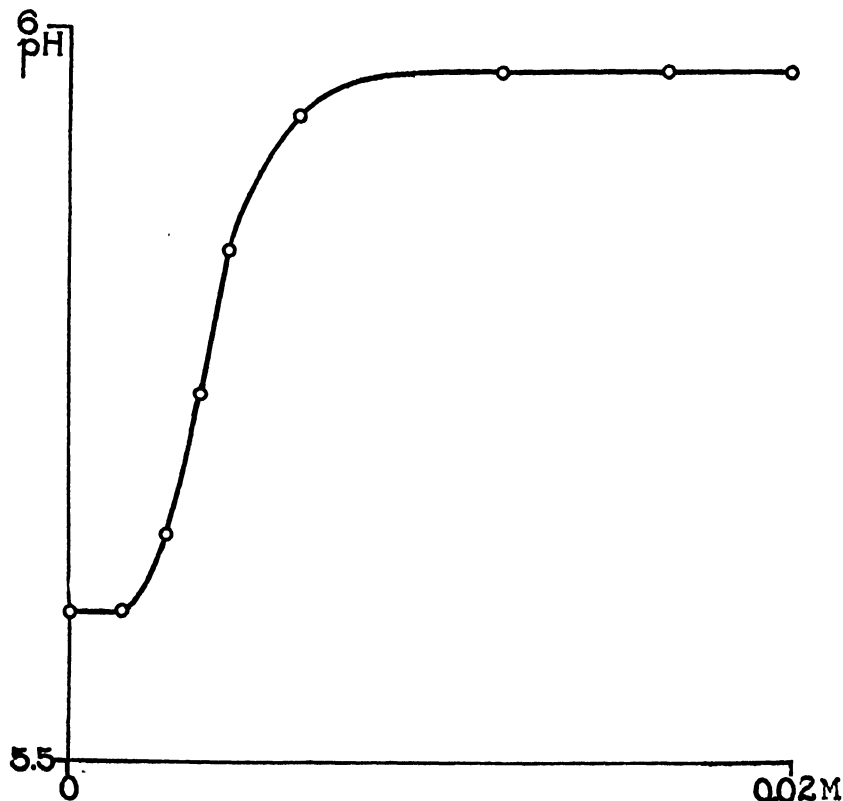
FIG. 5. Curve showing the relation between the concentration of NH_3 in the sap and the pH value of the sap. The ordinates represent the pH values and the abscissæ represent the concentrations of NH_3. The points on the curve are obtained from the curves as drawn in Fig. 3 (Curve A) and in Fig. 4.

the following is found to be true, as shown in Fig. 5. At the start the pH value of the sap remains unchanged until the concentration of NH_3 in the sap has reached 0.0014 M; this may be regarded as due to the buffer action of the sap (provided it is not NH_4Cl which enters the cell). Above this concentration the pH of the sap

increases but when the concentration of NH₃ in the sap has reached about 0.0064 M further accumulation of NH₃ brings about no appreciable change in the pH value of the sap until the cells die.

V.

Accumulation of the Dye in the Sap When the pH Values of the Sap Remain Constant While the Concentrations of NH₃ Are Varied.

When Figs. 3 and 4 are compared it is seen that after the cells have been placed in 0.005 M NH₄Cl solution at pH 6.9 $\left(\frac{M}{150}\right.$ phosphates$\left.\right)$ the accumulation of NH₃ in the sap takes place for about 5 minutes without a measurable change in the pH value of the sap. This enables us to carry out experiments in which we can compare the rate of accumulation of the dye in two lots of cells, one having no NH₃, and the other having 0.0014 M NH₃ in the sap (NH₃ is found to remain[12] in the sap during the experiment) while the pH value of the sap is the same in both cases.

Cells were placed in 0.005 M NH₄Cl solution at pH 6.9 $\left(\frac{M}{150}\right.$ phosphates$\left.\right)$ for 5 minutes after which they were removed, wiped, and placed in 0.00014 M dye solution at pH 6.7 $\left(\frac{M}{150}\right.$ phosphates$\left.\right)$, at $25 \pm 0.5°C$. and the rate of accumulation of the dye was measured at different intervals for 6 minutes. It was found that the rate (Fig. 6, Curve B) was slightly lower than that obtained in the case of the cells dipped for a few seconds in NH₄Cl and placed in the same dye solution. (Fig. 6, Curve A.) The decrease is about 24 per cent which was about the extent of decrease found at the end of 5 minutes when the cells were placed in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH₄Cl solution (Fig. 1, Curves A and B, in Section II). This indicates that the decrease is not due to the fact that NH₃ in the external dye solution hinders the entrance of the dye, but it was due to the presence of NH₃ in the sap. Since these

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[12] On removing the cells (containing 0.0014 M NH₃ in the sap) from 0.00014 M dye solution at pH 6.9, after 6 minutes, and testing the sap for NH₃, it is found that the NH₃ has not come out at all.

experiments were carried out when the cells had been placed in the NH$_4$Cl solution for only 5 minutes, we may assume that the cells were not injured at this stage.

It is of interest to see what happens when we employ cells whose
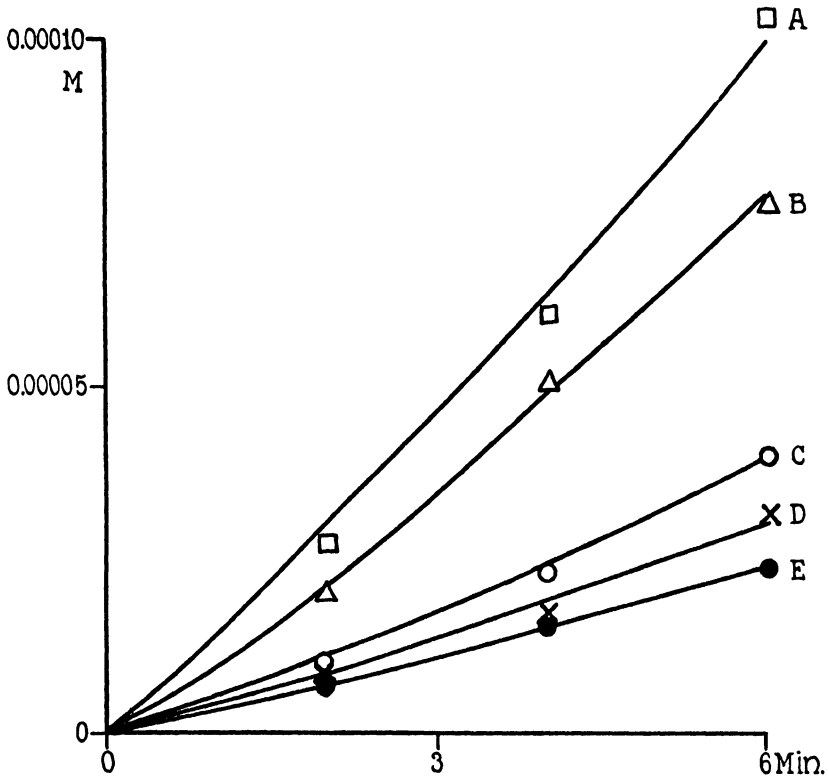
FIG. 6. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella* when the cells are placed in 0.00014 M dye solution at pH 6.7 after the cells have been treated for different lengths of time in 0.005 M NH$_4$Cl at pH 6.9. Curve A shows the rate when cells have been dipped for a few seconds in NH$_4$Cl; Curve B for 5 minutes; Curve C for 30 minutes; Curve D for 60 minutes, and Curve E for 120 minutes. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

sap has increased in alkalinity after a longer exposure, such as may possibly produce injury. The experiments in Sections III and IV show that the pH value of the sap is about 5.95, when the cells have been placed in 0.005 M NH$_4$Cl solution at pH 6.9 either for 30 min-

utes, 60 minutes, or 120 minutes though the concentration of NH₃ in the sap in the first case is 0.0064 M, in the second it is 0.0117 M, and in the third it is 0.0214 M. Such cells were removed from the NH₄Cl solution after 30, 60, and 120 minutes, wiped, and placed in 0.00014 M dye solutions at pH 6.7 $\left(\dfrac{M}{150} \text{ phosphates}\right)$ at 25 ± 0.5°C. When the rate of accumulation was measured at different intervals during 6 minutes (in this interval the concentration of NH₃ in the sap remained unchanged),[13] it was found that the rate decreased as the concentration of NH₃ increased in the sap as shown in Fig. 6, Curves C, D, and E. The rate of accumulation of the dye in the sap decreased about 24 per cent when there was 0.0014 M NH₃ in the sap, and when the pH of the sap was found to be 5.6˙(normal value). There was about 62 per cent decrease in the case of the cells which contained 0.0064 M NH₃ in the sap (pH value of sap was about 5.93). There was a decrease of about 71 per cent when the cells contained 0.0117 M NH₃ in the sap (pH value of the sap about 5.97). At this concentration of NH₃ in the sap the extent of decrease seemed to have almost reached its maximum since in the case of the cells containing 0.0214 M NH₃ in the sap at pH 5.97 there was only about 76 per cent decrease. This seems to indicate that the effect of NH₃ on the rate of accumulation of ·the dye in the sap reaches a maximum at a definite concentration of NH₃.

VI.

Further Experiments to Ascertain If NH₃ in the External Solution Hinders the Entrance of the Dye.

It has been indicated by the experiments described in Section V that NH₃ does not hinder the entrance of the dye unless NH₃ penetrates into the sap, but in order to confirm this the following experiments were made. When the cells have been placed in 0.005 M NH₄Cl at pH 6.9 $\left(\dfrac{M}{150} \text{ phosphates}\right)$ for 1 hour it is found that

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[13] When cells containing 0.0064 M, 0.0117 M, or 0.0214 M NH₃ in the sap are placed in 0.00014 M dye solution at pH 6.7 for 6 minutes, then removed, and the NH₃ of the sap determined, it is found that no NH₃ has come out of the sap.

0.0117 M NH₃ has accumulated in the sap; at this stage there is no measurable increase in NH₃ in the sap if the cells are left in the solution 6 minutes longer.

Cells were therefore placed in the NH₄Cl solution for 1 hour, removed, and wiped. One lot was now placed in 0.00014 M dye at pH
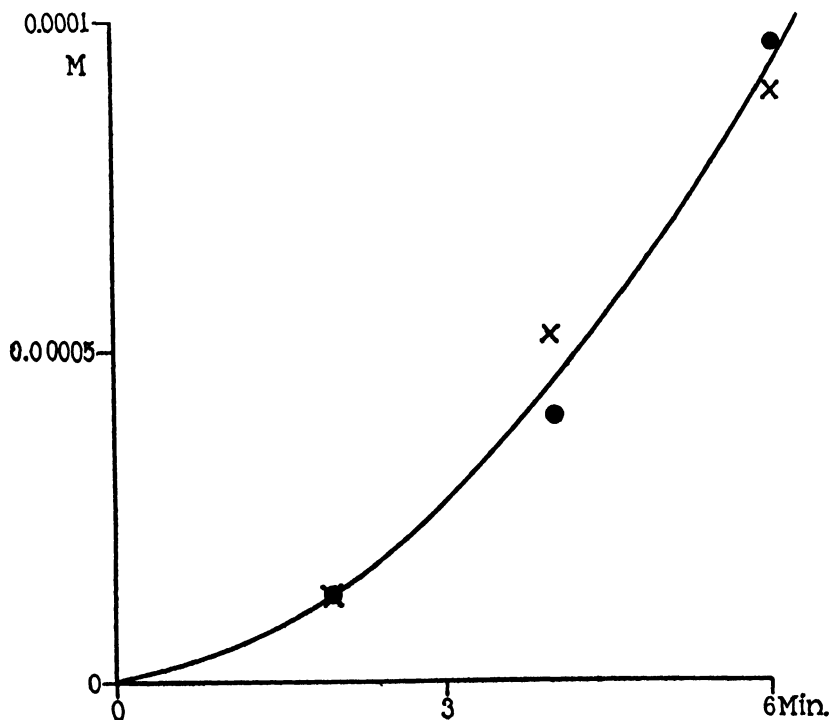
FIG. 7. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella* when cells are placed in a solution of dye after they have been placed for 1 hour in 0.005 M NH₄Cl at pH 6.9. The symbol (×) represents the rate of accumulation in 0.00014 M dye solution at pH 6.9, while the symbol (●) represents the rate in 0.00014 M dye solution at pH 6.9 containing 0.005 M NH₄Cl. Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

6.7 $\left(\dfrac{M}{150} \text{ phosphates} \right)$ and another lot in the dye solution of the same concentration and the same pH value containing 0.005 M NH₄Cl at 25 ± 0.5°C. When the rate of accumulation of the dye in the sap was measured at intervals during 6 minutes, it was found that

the rate was the same in both cases as shown in Fig. 7, symbol ● and symbol ✕. Thus it is evident that it is only the NH_3 in the sap which affects the accumulation of dye and that so long as this is constant variations to the above extent in the concentration of NH_3 in the external solution are of no import.

<div align="center">VII.</div>

Accumulation of the Dye in the Sap When the pH Values of the Sap Are Varied and the Sap Contains No NH_3.

Since it is evident from the experiments described above that the presence of NH_3 in the cell sap brings about a decrease in the rate of accumulation of the dye, the next step is to ascertain whether the increase in the pH value of the cell sap (in absence of NH_3) will have the same effect. Although we cannot obtain cells having the same concentrations of NH_3 in the sap, while the pH values of the sap are different, it is possible to change the pH values of the sap by placing the cells in solutions at pH 10.1 $\left(\dfrac{M}{40} \text{ boric acid } + \text{ NaOH mixtures.}\right)$ Fig. 3, Curve B shows that in this mixture the pH value of the sap increases but little in 30 minutes. This increase continues until the pH of the sap is changed from pH 5.6 to 5.9 in $2\dfrac{1}{2}$ hours after which there is very little change until the cells are dead.

Cells were placed in this mixture at 25 ± 0.5°C. for 5 seconds, 15, 30, 60, and 150 minutes, removed from the solutions, wiped, and placed in the 0.00014 M dye solution[14] at pH 6.7 $\left(\dfrac{M}{150} \text{ phosphates }\right)$ at 25 ± 0.5°C. An exposure of 15 minutes to the buffer solution at pH 10.1 caused a noticeable decrease in the subsequent accumulation

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[14] It is not possible to determine the pH value of the cell sap after the dye has entered the sap, hence another experiment was made. When the cells which had been in pH 10.1, as described in the text, for different lengths of time, were removed, and placed in pH 6.7 $\left(\dfrac{M}{150} \text{ phosphates }\right)$ at 25°C. for 6 minutes, it was found that the pH values remained the same. From this result it is assumed that the pH values of the sap will not change when cells are placed in the dye solution at pH 6.7 for 6 minutes.

of the dye and this decrease became greater the longer the cells were left in the solution at pH 10.1 (Fig. 8).
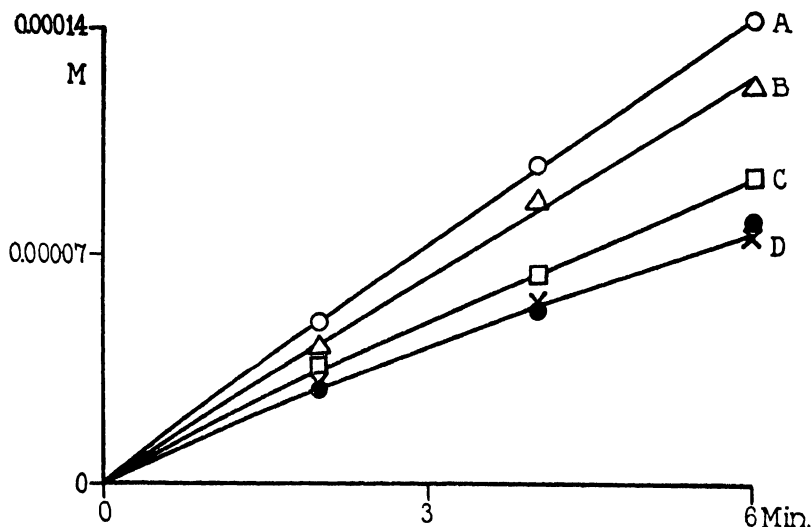
FIG. 8. Curves showing the rate of accumulation of dye in the sap of living cells of *Nitella* which have been placed in $\frac{M}{40}$ boric acid and sodium hydrate mixtures at pH 10.1 and then removed and placed in 0.00014 M dye solution at pH 6.7: Curve A, in the buffer solution for a few seconds; Curve B for 15 minutes; Curve C for 30 minutes; Curve D for 60 minutes (×) and 150 minutes (●). Each point on every curve is an average of forty experiments and the probable error of the mean is less than 7 per cent of the mean.

Fig. 8 shows that there may be a decrease of 13 per cent in the rate[15] of accumulation of the dye in the sap though the pH value of

[15] After the cells had been dipped in the $\frac{M}{40}$ buffer mixture of boric acid and sodium hydroxide at pH 10.1, wiped, and placed in 0.00014 M dye solution at pH 6.7 as already described, the rate of accumulation of the dye was greater than that found in the case of the cells which had been exposed in the same way to a buffer solution at pH 6.7 $\left(\frac{M}{150} \text{ phosphates}\right)$. When such cells were left for 2 minutes in the phosphate solution the rate was the same as in unwashed cells. Whether this increase is due to a preliminary stage of an injury, or to an adhering of the buffer mixtures to the surface of the cell, which cannot be readily washed out, and which produces an effect of the buffer mixtures on the surface of the cell apart from injury, cannot be stated definitely at present.

the cell sap remains normal (pH 5.6). There is a decrease of about 35 per cent when the pH value of the sap increases from 5.6 to 5.63. It is a striking fact that the decrease is only 44 per cent when the pH value of the sap reaches 5.7 or 5.9. From this it is evident that the decrease[16] in the rate may take place even when there is no change in the pH value of the sap, and that the extent of decrease reaches a maximum value when the pH of the sap is at 5.7. This may be due either (1) to the presence of substances, as in the case of NH_3, which compete with the dye for the combining substances in the sap, or (2) to injury to some part of the surface of the cell, which partly prevents the accumulation of the dye in the sap by allowing some to diffuse out of the cell, or (3) possibly to a combination of both.

VIII.

DISCUSSION.

The experiments described in the present paper show that there is a decrease in the rate of accumulation of the dye in the sap when NH_3 is present in the sap but that the presence of NH_3 in the external solution alone has no such effect.

The fact that NH_3 when present only in the external solution does not affect the entrance of the dye would seem to indicate that at the concentrations of the solution used there is no antagonism between NH_4^+ ions and D^+ ions in the sense that they might hinder each other from entering the living cell, and that there can be no tautomeric change in the dye brought about by NH_4Cl which could

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[16] When cells which had been placed in the $\frac{M}{40}$ boric acid and sodium hydroxide mixture at pH 10.1 for 2¼ hours were wiped, and placed in 0.005 M NH_4Cl at pH 6.9, 0.0007 M NH_3 was found to have accumulated in the sap in 5 minutes. This is much less than the concentration of NH_3 (0.0014 M) in the sap of a control cell placed directly in NH_4Cl solution at pH 6.9. When such cells were removed from the solution of NH_4Cl, wiped, and placed in 0.00014 M dye solution at pH 6.7 it was found that the rate of accumulation of the dye had decreased considerably as compared with cells which had been exposed to the buffer mixture for the same period but which had not been placed in NH_4Cl solution. The pH of the sap in both cases was about 5.9, so that the decrease was due to the presence of NH_3 in the sap.

produce such an effect. It is also evident that the dissociation of the dye is not affected by NH₄Cl, at the concentrations employed.

The decrease in the rate of accumulation of the dye in the sap may be interpreted as due to the fact that NH₃ and the dye compete for certain substances in the cell. The degree of competition as expressed by the decrease in the rate of accumulation of the dye may be dependent on the dissociation constants of the dye and of the NH₃ in the sap, and on the concentrations of these two substances. This is to be expected if we assume that the dye enters as DOH and, like NH₃, is capable of combining with weak acids and proteins in the sap. If the dye enters as a dye salt, *e.g.* DCl, and combines with a salt of a weak acid or of protein it may also be affected by the competition of NH₃ as already explained in the introduction.

Though it is not possible to determine experimentally at present, the same type of competition may exist in the protoplasm or in the surface membrane of the protoplasm, so that the assumption made in a previous paper[4] regarding the rôle of the surface membrane of the protoplasm may not be wrong.

It is evident from what has been said that it is not possible to determine experimentally the exact relation between the pH value of the sap and the decrease in the rate of accumulation which is found in the presence of NH₃.

Thus it is not yet possible to state definitely whether or not the dye enters the cell as the dye hydrate, but experiments are being carried on by the writer which may lead to a definite conclusion in the near future.

SUMMARY.

When the living cells of *Nitella* are placed in a solution of brilliant cresyl blue containing NH₄Cl, the rate of accumulation of the dye in the sap is found to be lower than when the cells are placed in a solution of dye containing no NH₄Cl and this may occur without any increase in the pH value of the cell sap. This decrease is found to be primarily due to the presence of NH₃ in the sap and seems not to exist where NH₃ is present only in the external solution at the concentration used.

THE PENETRATION OF CO_2 INTO LIVING PROTOPLASM.[*]

BY W. J. V. OSTERHOUT AND M. J. DORCAS.

(*From The Rockefeller Institute for Medical Research.*)

Experiments with H_2S on *Valonia*[1] indicate that under normal conditions it is chiefly if not exclusively undissociated molecules which enter the cell.[2] In view of the importance of this conclusion it seemed necessary to carry out comparable experiments with another substance. For this purpose CO_2 was chosen. It penetrates readily and is easily measured. Its importance in the organism renders its investigation particularly significant.

It might be supposed that the production of CO_2 by the cell would interfere with the measurements but it was found that under the conditions of the experiments this is so slight as to be negligible.

The experiments were carried out by the junior author, the methods being similar to those employed in the experiments on H_2S.[2] The CO_2, generated by the action of dilute HCl on Bermuda coral rock, passed through a column of cotton and bubbled through sea water until the latter had absorbed about one-fourth of its volume. The desired pH value was obtained by adding HCl or NaOH.

In each case 10 cells (the average volume of a cell being about 0.33 cc.) were placed in a bottle containing 125 cc. (with no air space above the solution) and tightly stoppered.

The process of penetration was practically complete in less than 2 hours but the cells were left for several hours in order to make sure that equilibrium had been attained. The solution was stirred during the exposure.

The sap was collected as described in the previous paper.[1] 1 cc. was used for each analysis. It was free from contamination by sea

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[*] Contributions from the Bermuda Biological Station for Research, No. 153.

[1] *Valonia macrophysa*, Kütz, collected at Bermuda.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131.

water as shown by the absence of SO_4. Sea water contains sufficient SO_4 to give a visible precipitate with acidified $BaCl_2$ when the sea water is diluted one hundred times. The absence of SO_4 also indicates that no injury has occurred and this is borne out by the fact that if cells which had been exposed to the experimental treatment were returned to sea water and kept under ordinary laboratory conditions they lived indefinitely.

The temperature ranged between 20° and 22°C. but did not vary more than 1° during any one experiment. The temperature coefficient (12.5° to 22.5°C.) of penetration and outward diffusion for living and dead cells is very low (less than 1.1).[3]

A Van Slyke apparatus[4] was employed to determine the total CO_2.

The essential question is whether the total CO_2 in the sap corresponds to the undissociated fraction of the total CO_2 in the sea water or to the dissociated fraction. The total CO_2 includes the HCO_3^- and CO_3^- ions, the undissociated H_2CO_3, the undissociated carbonates and bicarbonates of all the metals present, the uncombined or free CO_2, and an unknown number of hydrates and complexes of any one or all of the above.

If the concentration of total CO_2 inside the cell depends on the outside concentration of one of these substances, and is independent of the concentration of others, we should expect the ratio of concentration inside to that outside to change when we alter the proportion of this particular outside substance. There is no obvious method of changing only one of these substances. Changing more than one will serve our purpose provided that we do not change in the same ratio a substance which penetrates and one which does not, in such a way as to make it impossible to decide which substance penetrates.

The most expedient way is to change the H^+ ion concentration. An increase in the H^+ ion concentration would decrease the proportion of HCO_3^- and CO_3^- ions, and of other substances which change with them, and would increase the proportion of undissociated H_2CO_3 and of free CO_2.

[3] A low temperature coefficient for the absorption of pure gaseous CO_2 by solutions of K_2CO_3 was observed by Williamson, R. V., and Mathews, J. H., *Ind. and Eng. Chem.*, 1924, xvi, 1157.

[4] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Since we may assume that the relation between uncombined CO_2 and undissociated H_2CO_3 is approximately constant, we may for the purpose of the present paper (where relative values alone are considered) use the term undissociated H_2CO_3 to include the uncombined CO_2 (whose concentration is probably much greater than that of the undissociated acid). This course will be followed as a matter of convenience.

In order to ascertain the concentration of undissociated H_2CO_3 (including free CO_2) in the sea water at various pH values various means may be used. The method employed by McClendon is to determine the partial pressure of the CO_2 in the gas phase in equilibrium with the sea water (total CO_2 being constant). At low pH values, where the total CO_2 is undissociated, this partial pressure will reach its maximum value; as the pH value increases and H_2CO_3 begins to dissociate this value will fall in approximate proportion, e.g. when the total CO_2 is 50 per cent dissociated, the partial pressure will fall to about 50 per cent of its maximum value. The values of the partial pressures as determined by McClendon[5] are shown in Fig. 1 by the symbol ($\triangle$).

A somewhat different method (which gives similar results) was employed in the present investigation by circulating gas through sea water (at various pH values) and through artificial sap[6] (containing no excess base). If the pH value of the artificial sap is low enough to prevent dissociation of H_2CO_3, the amount of total CO_2 which passes over into the artificial sap corresponds to the undissociated H_2CO_3 (including free CO_2) in the sea water. For this purpose an apparatus was employed which consisted of two flasks fitted with tubes and an aspirator bulb so that the gas could be circulated and the two liquids brought to equilibrium with the same gas phase. The total CO_2 of the sea water and of the artificial sap was then determined with the Van Slyke apparatus. It was found that the lower the pH value of the sea water the higher was the relative total CO_2 of the artificial sap as compared with that of the sea water; this was true down to pH

[5] McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. 251*, 1917, 36. These values are for sea water of excess base 25 (at 20°C.).

[6] This was made by mixing 86.24 cc. of KCl 0.6 M with 15.08 cc. of NaCl 0.6 M; cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225.

3 below which lowering of the pH value produced no increase in the relative concentration in the artificial sap. The H_2CO_3 was therefore regarded as undissociated at pH values below 3. If the concentration of total CO_2 in the sea water is compared with that in the artificial sap, the latter is found to be about 16 per cent higher at pH values of 3 or lower, due to the fact that CO_2 is more soluble in the artificial sap. This is not surprising since CO_2 is less soluble in solutions containing sulfate.[7]

Let us suppose, for convenience, that the concentration of total CO_2 in the sea water is kept constant, for example at 100. Its concentration in the artificial sap (in equilibrium with the same gas phase) will be proportional to the concentration of undissociated H_2CO_3 (including free CO_2) in the sea water.[8] Thus if the pH of the artificial sap is low enough to prevent dissociation of H_2CO_3 its concentration of total CO_2 will be 116 when the total CO_2 in the sea water is undissociated; it will be 58 when the total CO_2 of the sea water is 50 per cent undissociated. Hence it is evident that we can find the percentage of undissociated H_2CO_3 (including free CO_2) in sea water by expressing the concentration of total CO_2 in the artificial sap as per cent of the total CO_2 in sea water and multiplying these figures by $100 \div 116$. Thus at pH 3 the per cent of undissociated H_2CO_3 (including free CO_2) in sea water is 100, at pH 5.8 it is 86 per cent, and so on.[9] The results are expressed in Fig. 1 by the symbol ($\times$).

These values may be compared with those which would be expected on a theoretical basis if the CO_2 were dissolved in distilled water. The undissociated H_2CO_3 (including free CO_2) expressed as per cent of total CO_2 may be calculated by means of the formula:[10]

$$\text{Per cent undissociated } H_2CO_3 \text{ (including free } CO_2) = \frac{100}{1 + \dfrac{K_1}{(H)} + \dfrac{K_1 K_2}{(H)^2}}$$

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[7] Cf. Hildebrand, J. H., Solubility, New York, 1924, 140.

[8] It is assumed that the apparent dissociation constant is the same in both.

[9] These determinations are approximate. It is probable that if care had been taken to keep the pH value of the artificial sap (at equilibrium) low enough to suppress ionization of H_2CO_3 in all cases there would be less irregularity.

[10] Cf. Michaelis, L., Die Wasserstoffionenkonzentration, 2nd edition, Berlin, 1922, 48.

in which $K_1 = 3.3 \times 10^{-7}$ and $K_2 = 6 \times 10^{-11}$. The values obtained by this calculation are expressed in Fig. 1 by the symbol ($\square$). The curve has the same general form as those already discussed but the latter are displaced somewhat to the left in the lower part. This is to be expected since E. J. Warburg[11] has shown that the presence of
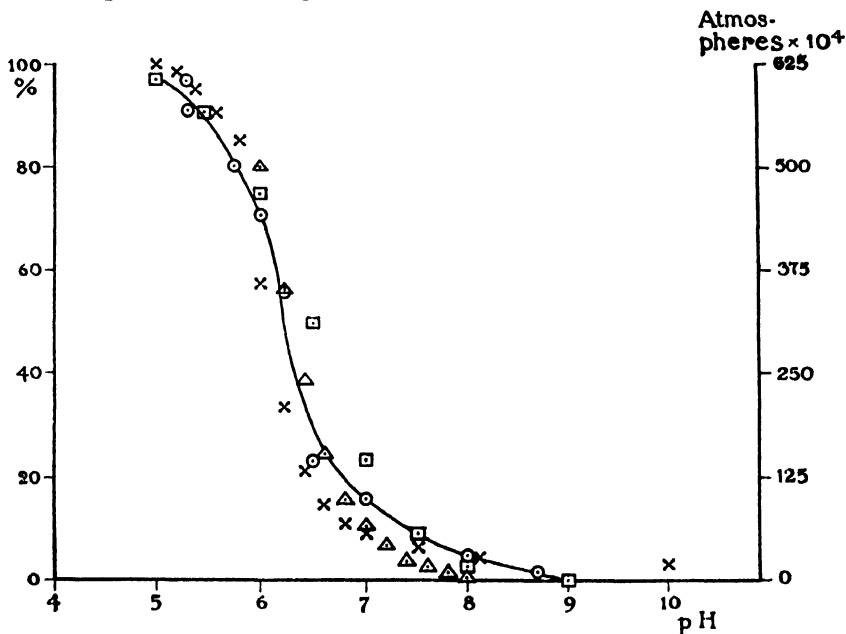
FIG. 1. Shows that the total CO_2 in the cell sap corresponds approximately to the undissociated H_2CO_3 (including free CO_2) in the sea water outside. The total CO_2 in the sap ($\bigcirc$) is expressed as per cent of that in the sea water outside (the curve is drawn free-hand through the points to give an approximate fit). The per cent of undissociated H_2CO_3 (including free CO_2) as calculated from the dissociation constant is shown by the symbol ($\square$). The partial pressure of free CO_2 in the sea water as determined by McClendon is shown by the symbol ($\triangle$). It is expressed as ten thousandths of a standard atmosphere as shown by the figures on the ordinate at the right. The concentration of H_2CO_3 (including free CO_2) in sea water, expressed as per cent of that found at pH 3 (where the H_2CO_3 is regarded as undissociated), is shown by the symbol ($\times$): it may be regarded as expressing the per cent of total CO_2 which is in the form of H_2CO_3 (including free CO_2). Each experimental point of symbols ($\square$) and ($\times$) represents one determination.

[11] Warburg, E. J., Biochem. J., 1922, xvi, 153. See also Van Slyke, D. D., Wu, H., and McLean, F. C., J. Biol. Chem., 1923, lvi, 765.

salts lowers the value of the negative logarithm of the apparent first dissociation constant of H$_2$CO$_3$: that this would shift the curve to the left is obvious.[10]   The relation of this to the theory of Debye and Hückel has recently been discussed by Hastings and Sendroy.[12]

Let us now enquire what takes place inside the cell.   If CO$_2$ acts in the same general way as H$_2$S and enters the cell only in the form of undissociated molecules we should expect the concentration of total CO$_2$ in the cell to correspond to the concentration of undissociated H$_2$CO$_3$ (including free CO$_2$) in the sea water.   This expectation is realized as is evident from Fig. 1 in which the circles denote the concentration of total CO$_2$ found in the sap of living cells at various pH values after equilibrium is reached between the total CO$_2$ inside and that outside.   The curve is drawn free-hand through these points to give an approximate fit.   In order to facilitate comparison the concentration of total CO$_2$ inside is expressed as per cent of that outside: by this method the correspondence (or lack of it) between the total CO$_2$ inside and the undissociated H$_2$CO$_3$ outside can be made most clearly evident.   If, for example, at a given pH value the undissociated H$_2$CO$_3$ (including free CO$_2$) in the sea water is about 50 per cent of the total CO$_2$ we shall expect the concentration of total CO$_2$ inside the living cell to be about 50 per cent of the total CO$_2$ in the sea water.   Hence the curve for per cent of undissociated H$_2$CO$_3$ (including free CO$_2$) in the sea water should approximately coincide with that for total CO$_2$ in the sap (expressed as per cent of undissociated H$_2$CO$_3$, including free CO$_2$, in the sea water).

Fig. 1 shows that the correspondence between the total CO$_2$ in the living cell (O) and the undissociated H$_2$CO$_3$ (including free CO$_2$) as shown by the symbols (X) and ($\triangle$) is fairly good.   It cannot be expected to be exact for a number of reasons.   In the first place, as already noted, CO$_2$ is more soluble in the sap than in the sea water.   In the second place, if we assume that H$_2$CO$_3$ penetrates freely but that its ions cannot pass in or out, it follows that if it partly dissociates after entering the cell the ions so formed will be trapped: more undissociated H$_2$CO$_3$ will move in, until the concentration of undissociated H$_2$CO$_3$ is the same inside and outside.   The total CO$_2$ inside, consist-

[12] Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxv, 445.

ing of undissociated H_2CO_3 (including free CO_2), plus ions, will therefore be greater than the undissociated H_2CO_3 (including free CO_2) outside. The amount of dissociation is not sufficient to make any great difference. If we regard the pH of the cell sap as approximately constant at 5.8,[13] we may, for purposes of calculation, take the per cent of dissociation calculated from the dissociation constant of CO_2 dissolved in distilled water; *i.e.*, about 19 per cent of the total CO_2. If the concentration of undissociated H_2CO_3 (including free CO_2) outside is 100 we should have the same concentration inside and this would be 81 per cent of the total CO_2 inside which would therefore amount to $(100 \div 81) 100 = 123.46$. The same relation would hold no matter what the outside pH or concentration of undissociated H_2CO_3 happened to be, *i.e.* we should always expect to find 23.46 per cent more total CO_2 inside than undissociated H_2CO_3 (including free CO_2) in the sea water outside. This excess will of course be less if the per cent of dissociation of H_2CO_3 in the sap is less.

Fig. 1 shows that at higher pH values there is more total CO_2 in the sap than undissociated H_2CO_3 (including free CO_2) in the sea water as calculated from the experimental data of McClendon and the writers. This would be expected on the grounds just mentioned (solubility and dissociation). At lower pH values, however, so much CO_2 might enter the cell as to lower the pH value, in which case the dissociation would be less and the total CO_2 of the sap would fall off somewhat. The curve indicates that this may be the case but at lower pH values the total CO_2 of the sap falls more than would be expected, becoming less than the undissociated H_2CO_3 (including free CO_2) of the sea water. For this no explanation is at present suggested.

Below pH 6.0 the curve for penetration becomes a little uncertain. The cells are soon injured at this pH and when they die the total CO_2 becomes the same inside and outside. Frequently we find that when the outside solution is at pH 5.8 or 6.0 the total CO_2 of the sap attains only 85 to 90 per cent of the outside concentration of total

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[13] The pH value of the sap varied but little except when lowered by the penetration of CO_2 and the difference in dissociation caused by this lowering may be neglected for our present purpose.

CO_2 even after standing 4 hours, as might be expected. Yet at other times we find practically 100 per cent with every evidence of normal condition of the cells. It is thought that this difference is not due to experimental error because there is no difficulty in getting consistent results with dead cells under these conditions.

In general it would seem that the concentration of total CO_2 inside the cell is approximately equal to the total undissociated H_2CO_3 (including free CO_2) outside. Possibly this would not be the case if the sap had much buffer action. The buffer action of the sap towards acids was found to be of a smaller order of magnitude than that of sea water. McClendon's excess base number[14] for this sea water was 24.8[15] for a typical sample and for the sap was less than 0.5.

Successive drops of 0.01 N HCl or $Ba(OH)_2$ were added to 1000 drops of sap, of distilled water, and of boiled KCl solution respectively, and the change in pH was measured by comparing with standard indicators. The sap has but little buffer action as is shown in Fig. 2. Its buffer action is of the same order of magnitude as that of a solution of KCl, of the same concentration of chloride from which most of the CO_2 had been driven off by boiling. According to the graph, 32 drops would change the sap from pH 8.0 to 6.2 and the sea water requires 200 drops for the same change.

We therefore seem justified in assuming that the total CO_2 inside the cell corresponds approximately to the total undissociated H_4CO_3 (including free CO_2) of the outside solution, and that the relations of the various ions in the sap are not complicated by the presence of any large amount of buffer.

If the facts found in the case of *Valonia* are generally valid we should expect that when the interior of any living cell is much more acid than the surrounding medium (the excess base being the same in both)

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[14] The excess base is determined by boiling off the CO_2 from the sea water and observing how much 0.01 N HCl must be added to 100 cc. of sea water to bring the pH back to the value it had before boiling: the number of cc. added is the excess base number. The excess base may be regarded as that part of the base which is not bound by strong acids and which in this case is largely bound by H_2CO_3. *Cf.* McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. 251,* 1917, 31.

[15] *I.e.* excess base = 0.00198 M.

the internal concentration of total CO_2 will be less than the external, providing the cell does not manufacture CO_2 rapidly enough to overcome the difference which would naturally exist at equilibrium. For example, in the case of *Valonia* we find that the total CO_2 content of the sap from cells in normal sea water (pH 8.2) is always less than that of the surrounding sea water. A typical analysis of cells that had
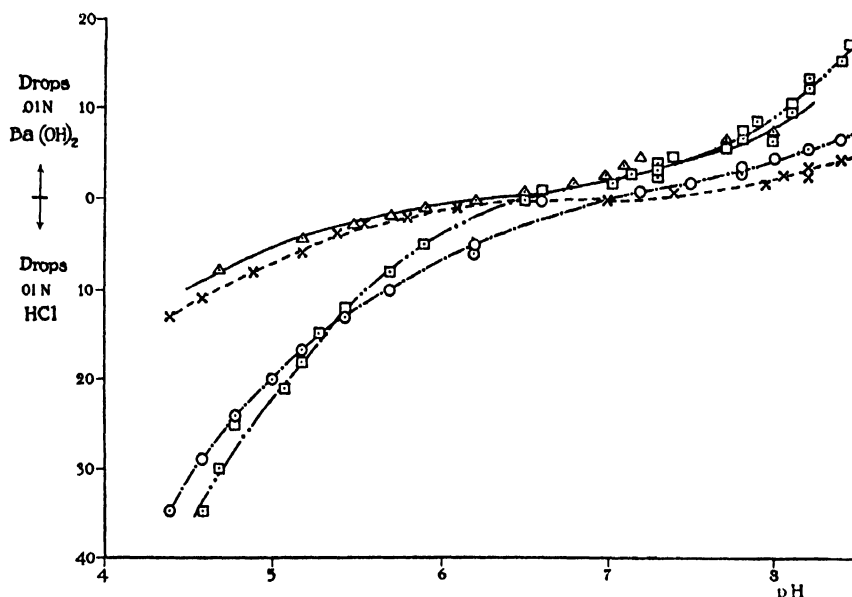
FIG. 2. Shows the buffer action of sap (□), of 0.6 M KCl (○), of boiled distilled water (×), and of unboiled distilled water from a wash bottle (△). The ordinates give the number of drops (of 0.01 N HCl or 0.01 N Ba(OH)₂) added to 1000 drops of the liquid. The curves are drawn free-hand to give an approximate fit. Each point represents one determination.

been 15 hours in a dark room showed that the sap had 0.009 cc. of total CO_2 per cc. of sap while the surrounding sea water had 0.038. A similar lot of cells after exposure to direct sunlight for 5 hours showed 0.004 cc. per cc. for the sap and 0.031 cc. per cc. for the sea water.

This has an important bearing on certain physiological problems in the study of which the assumption has frequently been made that the total CO_2 content of the cell at equilibrium is equal to that of the external medium.

Let us now consider another aspect of the penetration of CO_2 into *Valonia*. If we ignore the formation of CO_3^- ions and assume that some indiffusible ions are present so that a Donnan equilibrium is set up with H^+ and HCO_3^- diffusing in and out freely, but not undissociated H_2CO_3 or CO_2 we might assume the relation

$$\frac{H^+ \text{ inside}}{H^+ \text{ outside}} = \frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}}.$$
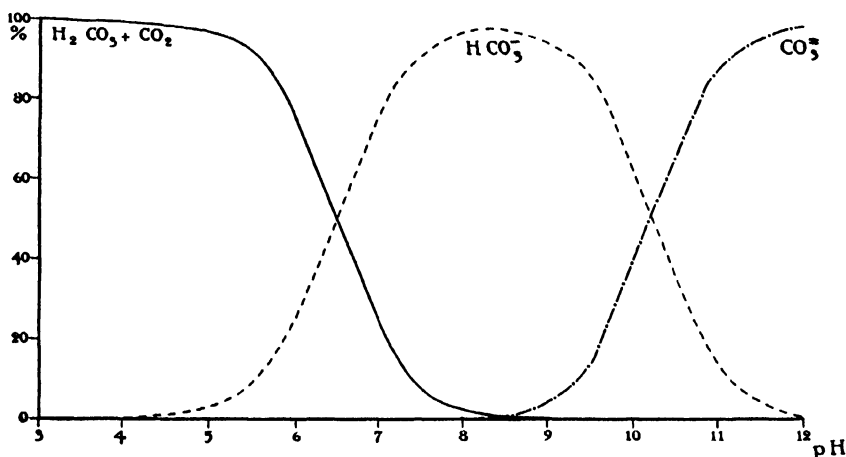
FIG. 3. Shows the per cent of undissociated H_2CO_3, including free CO_2, (———), of HCO_3^- (--------), and of $CO_3^=$ ($-\cdot-$) calculated from the formulæ:

$$\text{Per cent undissociated } H_2CO_3 \text{ (including free } CO_2) = \frac{100}{1 + \dfrac{K_1}{(H)} + \dfrac{K_1 K_2}{(H)^2}}$$

$$\text{Per cent } HCO_3^- = \frac{100}{1 + \dfrac{(H)}{K_1} + \dfrac{K_2}{(H)}}$$

$$\text{Per cent } CO_3^= = \frac{100}{1 + \dfrac{(H)}{K_2} + \dfrac{(H)^2}{K_1 K_2}}$$

in which $K_1 = 3.3 \times 10^{-7}$, and $K_2 = 6 \times 10^{-11}$.

All values are expressed as per cent of total CO_2.

Although this assumption may seem improbable,[16] it may be interesting to ascertain to what values it would lead.

If we regard the internal pH value as practically constant at pH 5.8 we should have, when the outside pH value is 6,

$$\frac{H^+ \text{ inside}}{H^+ \text{ outside}} \quad \frac{10^{-5.8}}{10^{-6}} \quad 1.59.$$

TABLE I.

A Comparison of $\frac{H^+ \text{ Inside}}{H^+ \text{ Outside}}$ *with* $\frac{HCO_3^- \text{ Outside}}{HCO_3^- \text{ Inside}}$ *at Various pH Values.*

It is assumed that the inside pH is constant at 5.8, and that $HCO_3^- = 19$ per cent of total CO_2 in sap.

pH inside.	pH outside.	Total CO_2 in sap.	HCO_3^- in sap.	HCO_3^- in sea water.	$\frac{H^+ \text{ inside}}{H^+ \text{ outside}}.$	$\frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}}.$
5.8	5.8	77	77(.19) =14.63	19	$\frac{10^{5.8}}{10^{5.8}} = 1$	$\frac{19}{14.63} = 1.299$
5.8	6.0	71	71(.19) =13.49	25	$\frac{10^6}{10^{5.8}} = 1.585$	$\frac{25}{13.49} = 1.85$
5.8	6.55	24	24(.19) =4.56	53	$\frac{10^{6.55}}{10^{5.8}} = 5.63$	$\frac{53}{4.56} = 11.62$
5.8	6.95	17	17(.19) =3.23	74	$\frac{10^{6.95}}{10^{5.8}} = 14.13$	$\frac{74}{4.07} = 22.9$
5.8	8.0	5	5(.19) =0.950	97.3	$\frac{10^{8.0}}{10^{5.8}} = 158.5$	$\frac{97.3}{0.950} = 102.4$

From Fig. 1 we find that the total CO_2 in the sap at pH 6 = 71 (regarding the total CO_2 in the sea water as constant at 100). For the purpose of comparing the HCO_3^- in sap and sea water at the pH values here considered we may assume that all the CO_2 is H_2CO_3 (in ionized or non-ionized form) and consider that 19 per cent of the total CO_2 in the sap (Fig. 3) is ionized at pH 5.8: we then have as the ionized portion (71) (.19) = 13.49. From Fig. 3 we learn that all of this may

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[16] Some objections to it have been stated in a previous paper.[2] The fact that the pH value of the sea water has been varied within wide limits (by adding HCl or NaOH) with little or no effect on the pH value of the sap indicates that H$^+$ ions do not diffuse in and out freely.

be regarded as HCO_3^-. In the sea water we have at pH 6 (Fig. 3) $HCO_3^- = 25$. We therefore have

$$\frac{HCO_3^- \text{ outside}}{HCO_3^- \text{ inside}} = \frac{25}{13.49} = 1.85.$$

Proceeding in this manner we obtain the values given in Table I. The figures in the last two columns increase in somewhat the same fashion and the deviations are such as might result largely from experimental errors. The greatest discrepancy occurs where the total CO_2 inside deviates most from the values calculated from the dissociation constant (see Fig. 1).

It is evident that if it is the ions alone which enter, the rate of penetration will increase as the pH value of the external solution increases, while if it is only the undissociated molecules which enter, the rate of penetration will increase as the pH value of the sea water falls. The latter is found to be the case, and this would indicate that it is the undissociated molecules which enter unless for some reason the rate of penetration is proportional, not to the concentration of the penetrating substance in the sea water, but to its concentration at equilibrium in the sap, which appears improbable. It is of course possible that both ions and undissociated molecules enter but that the latter penetrate much more rapidly.

In order to arrive at the conclusion that ions enter, we are obliged to make improbable assumptions, and unpublished electrical experiments by L. R. Blinks make it difficult to believe that ions are able to penetrate. We may therefore conclude that little or no CO_2 enters except in the form of undissociated molecules.

In this connection it may be noted that the work of Loeb,[17] Harvey,

[17] Loeb, J., *Biochem. Z.*, 1909, xv, 255; 1910, xxiii, 95; *Arch. ges. Physiol.*, 1897–98, lxix, 1; 1898, lxxi, 457; Artificial parthenogenesis and fertilization, Chicago, 1913, 143; *J. Gen. Physiol.*, 1922–23, v, 231. Harvey, E. N., *Internat. Z. physik.-chem. Biol.*, 1914, i, 463; *Carnegie Institution of Washington, Pub. 212*, 1915. Crozier, W. J., *J. Gen. Physiol.*, 1922–23, v, 65, with references to earlier papers. Haas, A. R. C., *J. Biol. Chem.*, 1916, xxvii, 225. Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; 1920, liii, 457; *Biol. Bull.*, 1922, xlii, 14. Brooks, M. M., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 1449, 1470. Beerman, H., *J. Exp. Zool.*, 1924–25, xli, 33. Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

Crozier, Haas, Jacobs, M. M. Brooks (dealing with *Valonia*), Beerman, Clowes, Smith, and others, on CO_2 and on various weak acids, indicates that undissociated molecules penetrate, although the methods employed do not enable us to decide positively whether ions enter or not.   Those who have concluded that ions do not enter have done so on indirect grounds.   This is also true to some extent where the opposite conclusion has been reached (*cf.* Smith and Clowes, and Van Slyke, Wu, and McLean).[18]

SUMMARY.

The experiments indicate that little or no CO_2 enters normal cells of *Valonia* except in the form of undissociated molecules.

Whenever the interior of a cell is more acid than the surrounding medium (excess base being the same in both) we may expect that at equilibrium the internal concentration of total CO_2 will be less than the external.

[18] Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183.   Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

GROWTH AND PERSISTENCE OF FILTERABLE VIRUSES IN A TRANSPLANTABLE RABBIT NEOPLASM.

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Observations concerning the growth and persistence in a transplantable rabbit tumor of two filterable viruses, Virus III and vaccine virus, are reported in this paper.

In 1923 Levaditi and Nicolau (1) reported observations concerning the growth and survival of vaccine virus in neoplasms of mice and rats. Evidence favoring his theories of dermotropism and neurotropism of viruses was considered by him to be found in the fact that vaccine virus does not grow or survive in mouse or rat sarcoma but does multiply in mouse cancer and survives throughout the life of the neoplasm. Among other conclusions he stated that cancer cells are unable to develop an active immunity against infection with vaccine virus and only passively partake of their host's immunity.

Levaditi's observations and conclusions are interesting and, if confirmed, are of importance in regard to the growth of viruses in general and, also, in regard to the biology of tumor cells, particularly their immunological response to infection. A unique opportunity for the study of certain problems of this nature is afforded by our possession of a virus and of a transplantable neoplasm of the same animal species—the rabbit. In beginning this work it was decided to determine, first, if the virus would grow in the tumor, and if so what effect this condition would have upon the character of the malignant disease. The present paper is concerned primarily with the first of these questions.

Methods and Materials.

Virus III.—While attempting to produce chicken-pox in rabbits a filterable transmissible pathogenic agent was discovered (2). This agent produces gross as well as microscopic lesions in the cornea, skin, and testicles of rabbits, and an

infection with it leads to an immunity against subsequent infections with the same material. For convenience this agent has been spoken of as Virus III. The virus has been transferred from rabbit to rabbit at 3 to 5 day intervals over a period of 2½ years. It acts like a fixed virus in that a small amount of it when injected into the testicles of rabbits causes a sharp rise of temperature to 104–106°F. 3 to 5 days after the inoculation, and because intradermal injections of the virus in dilutions of 1:1000 are regularly followed by visible skin reactions. Although the virus can be recovered from the heart's blood of rabbits following intratesticular or intradermal inoculations, it does not cause death even when injected intracerebrally. The testicular emulsions containing the virus have been shown repeatedly to be free from ordinary anaerobic and aerobic bacteria by means of cultures on blood agar, in broth, and in Smith-Noguchi tubes. Furthermore, ordinary bacteria have not been seen in stained films and dark-field preparations of the emulsions containing the virus, in stained sections of inoculated testicles, or in sections of inoculated testicles impregnated with silver nitrate. Local infiltration of the tissues with endothelial leucocytes, swelling of the involved epithelial cells, and the presence of nuclear inclusions in both the endothelial leucocytes and the epithelial cells are the characteristic pathological changes observed in rabbits experimentally inoculated with the virus. At first it was considered not unlikely that the virus is the etiological agent of varicella. Further work, however, in our laboratory and also in Swift's (3), disclosed the fact that Virus III is indigenous to rabbits. From the work completed, it seems most likely that Virus III is as typical a virus as vaccine virus or the virus of symptomatic herpes from both of which it can easily be differentiated.

In the experiments to be reported, the presence or absence in rabbits of an immunity to Virus III was determined by means of intradermal inoculation of the virus or by testing the virucidal properties of the sera.

Vaccine Virus.—The vaccine virus used in these experiments was obtained from Noguchi (4) and is a strain which has been propagated during a number of years by frequent passage from rabbit to rabbit by testicular inoculation. Inoculation of the scarified skin or cornea of rabbits is followed by typical macroscopic and microscopic lesions. The presence or absence in rabbits of an immunity to the virus was determined by intradermal inoculation of it.

Neoplasm.—A number of papers dealing with the tumor employed in these experiments have already been published (5). Suffice it to say that the tumor arose in the scrotal skin of a rabbit which eventually showed marked constitutional symptoms and finally succumbed with a widespread distribution of metastatic growths. Successful transplantation from the original animal through successive generations was accomplished by intratesticular inoculations (6). The original growth appeared to be of epithelial origin, composed of cells allied to those found in the bulb and root sheath of the hair. The general morphology of the cells and the structure of the growth have been unaffected by transplantation.

Although various routes of inoculation may be used with which a primary growth and, at times, metastases are obtained, it has been found that intratesticular

inoculation is most satisfactory from the point of view of an actively developing and persistent primary tumor and for the study of the disease process as a whole. As a routine procedure, a group of not less than ten rabbits is inoculated each month from an actively growing primary tumor of the previous month's series. In the present paper tumors from such groups are referred to as "stock tumors". The general character of the disease in different groups fluctuates from time to time, and, furthermore, there are well defined differences in the disease picture of individual animals in any single group.   Such factors as the growth and fate of the primary tumor, the animal incidence of metastases, their number, extent, and distribution, and the actual and probable mortality rates are significant variables in this connection.   However, for the purpose of the present paper, it is sufficient to say that an actively progressing primary tumor is obtained by intratesticular inoculation in practically every instance and that by the 14th day after inoculation the growth has frequently replaced the entire testicle.   In a certain number of rabbits the primary tumor continues to grow, involving the cord and extending into the abdominal cavity; in others regression, absorption, and eventual healing occur, while some animals continue to carry a large primary tumor composed of both living and necrotic tissue for many weeks.

From an analysis of the postmortem examinations of the first twenty generations of the tumor (7), the mean incidence of secondary growths for the entire group of animals was 60 per cent and upon the same basis the average mortality varied from 20 to 40 per cent.   Metastases have been found in practically every organ and tissue of the body, the most common sites being the kidneys, lungs, suprarenals, liver, eyes, and the serous membranes of the abdomen.   There occur, also, cord and retroperitoneal tissue extensions from the primary growth.   Gross metastases have been found as early as 2 weeks after inoculation and there is no doubt that emboli of tumor cells are distributed soon after inoculation of the testicle.   Death from extensive and widely distributed tumor growth has occurred as early as 3 weeks after inoculation and in some instances actively growing metastases were still present in animals killed as late as 7 months after inoculation. On the other hand, some rabbits whose primary tumor attains large proportions are able completely to suppress and eventually to heal this growth and at autopsy no signs of secondary tumors are to be found.

The characteristic feature of variation in the disease, as shown by individual animals of the same group and by different groups of animals, has been considered highly significant from the point of view of animal reaction and animal economy. It should be emphasized that in any study of the effect which various agents, such as the filterable viruses, may have upon the growth and malignancy of the tumor, one must take into account this biological characteristic of the disease. This phase of our study is reserved for a further report.

Growth and Survival of Virus III in the Tumor.

A number of young actively growing tumors were inoculated with Virus III to determine, first of all, if the virus would grow and persist in·the tumor.   Details of these experiments will be omitted since early in the work we were confronted with the fact that all the stock tumor rabbits, into which no virus had been intentionally inoculated, became immune to infection with Virus III.   Table I summarizes a few of the experiments showing the development of an immunity in the stock tumor rabbits to Virus III.   A comparable number of animals inoculated with *Treponema pallidum* or *Treponema pertenue* and housed under conditions similar to those of the tumor rabbits were used as controls.   ·

The immunity of the stock tumor rabbits to Virus III gains in significance when it is realized that the stock tumor is transferred only at monthly intervals, and that all of the animals develop immunity to the virus 2 to 3 weeks after inoculation with the tumor.   Several possible explanations for this refractory state occurred to us; namely, (1) that the virus, accidently introduced into the tumor, multiplies and survives longer in it than in testicular tissue and was being transferred each month with the tumor, (2) that the tumor at one time accidently infected with the virus had itself acquired an immunity which it conferred upon all its subsequent hosts, or (3) that the tumor in some non-specific manner protected its host against infection with the virus.

In proceeding to investigate this question, attempts were first made to demonstrate the presence of Virus III in the stock tumor by means of rapid passage of emulsions of the primary tumor or metastases through several series with not more than three successive rabbits in each.   The tumor tissue selected for these experiments appeared, on gross examination, to be living for the most part.   It will become obvious further on in the report why more than three passages in each series were not made.   By means of the procedure it was possible to demonstrate regularly the presence of Virus III not only in the primary tumor but also in metastases.   54 days was the longest interval elapsing between the intratesticular inoculation of the tumor and the

TABLE I.

Summary of Experiments Showing that the Inoculation of Rabbits with Stock Tumor Was Followed by a Refractory State of the Animals to Virus III.

Experiment No.	No. of rabbits.	Test for immunity to Virus III before inoculation.	Inoculation.	Site of inoculation.	Interval between inoculation and test for immunity to Virus III.	Method of testing for immunity to Virus III.	Result.
					days		
I	5	Not made.	Tumor.	Skin.	24	Intradermal inoculation of Virus III.	All immune.
II	5	" "	" "	"	49	" "	" "
III	8	" "	" "	Right testicle.	61	" "	" "
IV	3	" "	" "	" "	45	Virucidal properties of sera.	All virucidal.
V	5	" "	" "	" "	14	" "	" "
VI	10	None of the sera virucidal for Virus III.	" "	" "	25	" "	" "
VII	20	Not made.	Syphilis or yaws.	" "	mos. 3–12	Intradermal inoculation of Virus III.	None immune.

The action of Virus III was controlled in each instance either in the skin of normal and immune rabbits or by virucidal tests with sera from normal and immune rabbits.

test for the presence of the virus in the primary growth and metastases. In Text-fig. 1 some of the experiments which demonstrate the presence of Virus III in the primary and metastatic growths are graphically illustrated.

Although inoculation of stock tumors, 4 to 8 weeks old, into the testicle led to an immunity in rabbits to Virus III, no visible virus reaction was obtained by means of intracutaneous inoculations of the same material. From previous work with Virus III it has been shown that amounts of virus too small to give visible skin reactions are sufficient to immunize the animals and that these small amounts of virus are sufficiently increased after two or three testicular passages to produce visible reactions. From the experiments thus far reported, one may conclude that Virus III is regularly present in stock tumors, 4 to 8 weeks old, in quantities sufficient to immunize rabbits but not great enough to produce visible skin reactions. As we were interested not only in the presence but also in the survival of the virus in the neoplasm, it was essential that we work with older tumors. Nevertheless, we were also interested in determining if intracutaneous inoculations of young stock tumors would produce visible Virus III reactions. A number of stock tumors, 7 to 10 days old, were removed from testicles or skin, emulsified, and tested under properly controlled conditions for their ability to produce visible Virus III reactions in the skin of normal rabbits. It was found that intracutaneous inoculations of such emulsions were followed by typical virus reactions (Text-fig. 1). In this connection it is of interest and of probable significance to note that nuclear inclusions, typical of Virus III reactions, were found in the young stock tumors. Similar inclusions, on the contrary, have not been seen in older tumors nor in a strain of the tumor free from the virus which will be described later in the paper.

The results of the work thus far show that Virus III is present in the stock tumor and is being transferred with it each month, that it multiplies in the tumor and survives there for at least 54 days. Since it has been shown that rabbits inoculated with the stock tumor themselves became immune to Virus III 2 or 3 weeks later, the survival of the virus in the tumor for 8 weeks evidently takes place despite the immune state of the rabbit host.

Growth and Survival of Virus III in a Transplantable Rabbit Tumor.

Oct. 15, 1924, right testicle of Rabbit A inoculated with 0.3 cc. stock tumor emulsion. Tumor grew and metastasized.

Dec. 8, 1924, *54 days later,* rabbit sacrificed and portions of primary tumor and metastases in retroperitoneal, axillary, mesenteric, and mediastinal lymph glands were injected into stock rabbits as indicated below.
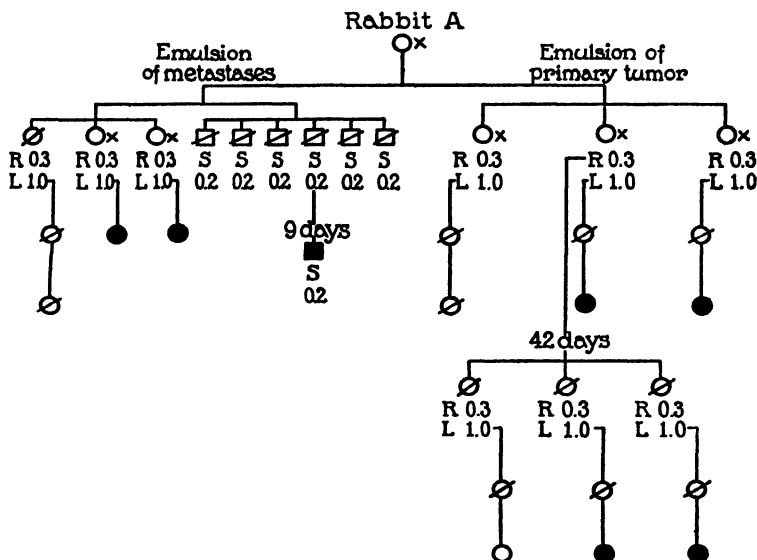
Rabbit A

Emulsion of metastases

Emulsion of primary tumor

R 0.3 L 1.0 R 0.3 L 1.0 R 0.3 L 1.0 S 0.2 S 0.2 S 0.2 S 0.2 S 0.2 S 0.2 R 0.3 L 1.0 R 0.3 L 1.0 R 0.3 L 1.0

9 days

S 0.2

42 days

R 0.3 L 1.0 R 0.3 L 1.0 R 0.3 L 1.0

Each circle represents one rabbit.

R indicates right testicle; L, left testicle; S, skin. Figures following R, L, and S indicate amounts in cc.

Passages from left testicles to testicles of subsequent animals were made at 4 day intervals. Further passages from rabbit to rabbit were made at 4 day intervals by means of bilateral testicular inoculations of testicular emulsions. Intradermal inoculations were made in the majority of the rabbits. Passage from R was made from tumor in right testicle.

● indicates that a visible Virus III lesion was noted in the skin, that the testicles contained a virus which was identified as Virus III, and that the animal was immune 3 weeks later to Virus III inoculated intradermally.

∅ indicates absence of gross virus lesions; rabbit immune to Virus III 3 weeks later.

○ indicates absence of gross virus lesions; rabbit not immune to Virus III 3 weeks later.

× indicates that immunity of the rabbit to Virus III was not tested.

⌀ indicates a female rabbit inoculated intradermally with tumor emulsion and immune to Virus III 3 weeks later.

■ indicates a visible Virus III lesion in skin of female rabbit produced by emulsion of tumor removed from skin of another female rabbit. Test controlled in the skin of a rabbit immune to Virus III.

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Growth and Disappearance of Virus III Injected into the Testicles of Normal Rabbits.

It seemed that 4 to 8 weeks is an unusually long time for Virus III to survive in the tumor. From previous work it had been found that Virus III injected into the testicles of normal rabbits multiplies rapidly, probably reaching the maximum growth between the 4th and 6th days. The virus also disappears rapidly from the testicles and frequently no visible reaction is obtained in the skin with emulsions of testicles removed 8 days after inoculation. The mere fact that no visible reaction is obtained is not adequate proof that all the virus is dead, and, in view of the long survival of the virus in the tumor, it seemed essential to investigate its length of survival in the previously normal animal after intratesticular inoculation. Consequently, the following experiments were performed to determine when one can be reasonably certain that the virus is no longer alive in the experimentally infected rabbit.

Rabbit 1 received 1.0 cc. of a testicular emulsion containing active Virus III in each testicle and 0.2 cc. in the skin. The usual virus reaction occurred in the skin and testicles. 34 days later the animal was etherized and both testicles and the inguinal, popliteal, and axillary lymph glands were removed, emulsified, and injected into the testicles of three normal rabbits. A month later these rabbits were tested for the presence of an immunity to Virus III by means of intracutaneous inoculations of the virus and all were found to be susceptible.

Rabbit 2 was inoculated in a manner similar to Rabbit 1. Both testicles were taken out under ether anesthesia 4 days later. 30 days after inoculation the inguinal, axillary, and popliteal lymph glands were removed, emulsified, and injected into the testicles of two normal rabbits. A month later these rabbits were tested for the presence of an immunity to Virus III and were found to be susceptible.

The above experiments afford indirect evidence that Virus III is no longer present in the testicles and lymph glands of the experimentally infected rabbit a month after the inoculation or, if present, that it exists in such small quantities or in such condition as to be incapable of inducing immunity. It seemed advisable to pursue the matter further by means of rapid passage of the emulsions of lymph glands and testicles from experimentally infected animals through several series of not more than three successive stock rabbits in each to obtain,

if possible, some visible or immunological evidence concerning the survival of the virus. A month was the period of time arbitrarily chosen to elapse between the inoculations and the attempts to demonstrate a viable virus in the experimentally infected rabbits. Three experiments were conducted as outlined and after the passages had been made all the animals were held for 3 or 4 weeks, after which time they were tested for the presence of an immunity to Virus III.

It must be remembered in connection with these experiments that Virus III is indigenous to rabbits and that it was first discovered by the serial transfer, at 3 or 4 day intervals, of rabbit's testicles which in the first instance had been inoculated with blood from chicken-pox patients. In the work on varicella, macroscopic evidence of Virus III was noticed only once in twelve series earlier than the fourth passage. If the passages were continued further, macroscopic reactions became numerous for the reason that each passage increased the chances of encountering a rabbit spontaneously infected with the virus and of concentrating the virus sufficiently to produce visible reactions. In the light of this experience, it is obvious why not more that three successive rabbits were employed in a series in the experiments reported in the present paper. In connection with the tests for the presence of an immunity in the rabbits held 3 or 4 weeks, as mentioned above, it must also be remembered that a certain number of stock rabbits are immune to Virus III. Since the virus is indigenous to rabbits, this condition is to be expected. The percentage of immune animals from miscellaneous stocks and from a number of different sources has been variable, but a fair average is 15 to 20 per cent.

Examination of the results of the experiments graphically illustrated in Text-fig. 2 reveals that only one visible Virus III skin reaction was obtained in ten series and that only 20 per cent of the first rabbits to be inoculated in the ten series were immune to Virus III 3 weeks after inoculation. Since these results coincide with those obtained in previous work in which the series were begun with human blood, one may be reasonably certain that Virus III is not present in the testicles and lymph glands of experimentally infected rabbits a month after inoculation. A comparison of the results of the experiments with the stock tumor (Text-fig. 1) with those shown in Text-fig. 2 reveals that Virus III survives much longer in the tumor than it

Absence of Virus III in Lymph Glands and Testicles of Rabbits 1 Month after Inoculation.

Nov. 1, 1924, skin and testicles of Rabbit A were inoculated with Virus III. Marked reaction in skin and testicles.

Dec. 4, 1924, *33 days later,* both testicles and axillary, popliteal, and inguinal glands removed, emulsified, and injected into testicles of stock rabbits as indicated below.

Experiment I
Rabbit A

Glandular emulsion — Testicular emulsion

Experiment II
Rabbit B

Emulsion of lymph glands and testicles 33 days after inoculation of virus III into skin and testicles

Experiment III
Rabbit C

Emulsion of lymph glands and testicles 33 days after inoculation of virus III into skin and testicles
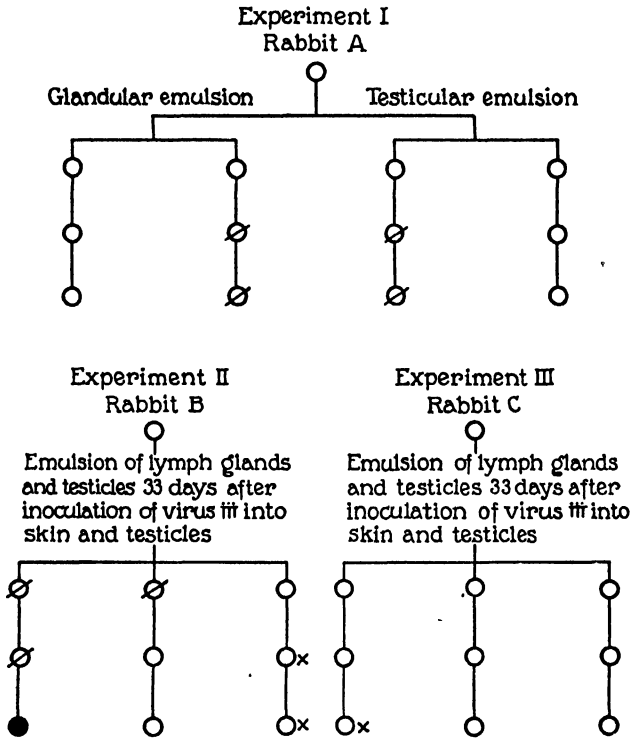
Each circle represents one rabbit.

Passage from animal to animal was made by means of testicular emulsions injected into testicles of stock rabbits at intervals of 4 days. The skin was also inoculated in each rabbit.

● indicates that a visible Virus III lesion was noted in the skin, that the testicles contained a virus which was identified as Virus III, and that the rabbit was immune 3 weeks later to Virus III inoculated intradermally.

∅ indicates absence of gross virus lesions.. Rabbit immune to Virus III 3 weeks later.

O indicates absence of gross virus lesions and that the rabbit was not immune to Virus III 3 weeks later.

✕ indicates that immunity of the rabbit to Virus III was not tested.

does in testicular tissue. The reasons for the unusually long survival of the virus in the tumor are being investigated at present.

Strain of Tumor Free of Virus III.

It is not known when the tumor became infected with Virus III. After it was found that the tumor carries the virus, the question naturally arose as to whether the character of the tumor is affected by its presence. Previous work has shown that the injection of Virus III alone does not produce tumors. It was not known, however, whether the presence of the virus affects the growth of the tumor cells in such a way as to influence the malignancy of the disease. To determine what effect the virus has upon the tumor it was essential to obtain a strain of the tumor free of the virus. The work with the stock tumor, bearing the virus, led us to believe that this would be difficult of accomplishment inasmuch as the virus survives in the tumor many weeks after the rabbit host becomes refractory to skin infection with the virus and after its blood serum is highly virucidal. Nevertheless, an accident enabled us to obtain a strain of the tumor entirely free of Virus III.

A rabbit inoculated with the stock tumor bearing the virus died several weeks later and accidently lay in a warm room 12 to 18 hours. The primary tumor was removed and inoculated into the testicles of three rabbits. It was fully 3 weeks before one could be sure that the transplants were increasing in size. Growth, once initiated, progressed, however, and transfers were successfully made from one of the tumors. A number of generations of this strain of the tumor have been studied and we are convinced that Virus III is no longer present in it inasmuch as it has been impossible to demonstrate its presence (Text-fig. 3) by methods which proved successful with the stock tumor. Moreover, animals inoculated with this strain do not become refractory to skin infection with Virus III and their sera do not become virucidal (compare Text-figs. 1 and 3). In addition, no nuclear inclusions, typical of Virus III reactions, have been found in young virus-free tumors.

These findings show conclusively that the virus is not essential for the growth of the tumor inasmuch as the strain of tumor free of Virus III grows, metastasizes, and causes death. Further than this one cannot go at present. The behavior, however, of the tumor bearing the virus in comparison with that of the tumor without the virus is being studied.

Text-Fig. 3.

Strain of Transplantable Rabbit Tumor Free of Virus III (a Virus-Free Strain of Transplantable Rabbit Tumor).

Oct. 15, 1924, right testicle of Rabbit A inoculated with 0.3 cc. of same stock tumor emulsion used in inoculating Rabbit A in Text-fig. 1. Tumor grew.

Nov. 13, 1924, rabbit died and lay in a warm room 12 to 18 hours. Primary tumor removed, emulsified, and inoculated into stock rabbits as indicated below.
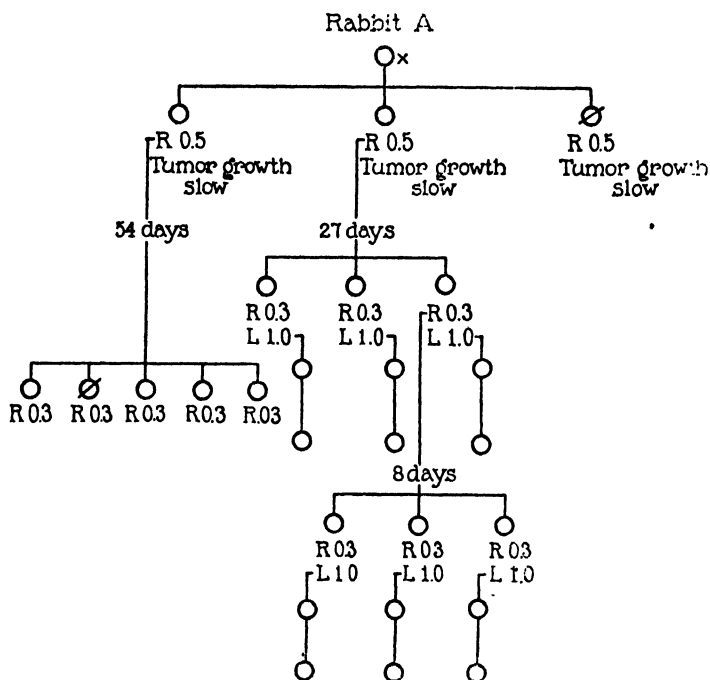
Procedure and symbols same as those in Text-fig. 1.

Growth and Survival of Vaccine Virus in the Tumor.

When it was found that Virus III grows in the rabbit tumor and survives there for an unusually long time, it seemed probable that confirmatory or additional facts in regard to the problem under study might be obtained by similar experiments with vaccine virus. Therefore, a young tumor was inoculated with vaccine virus, removed 4 weeks later, and passed to several normal rabbits by means of intratesticular inoculations. The tumor was experimentally infected only

Growth and Survival of Vaccine Virus in a Transplantable Rabbit Tumor.

Oct. 15, 1924, right testicle of Rabbit A inoculated with tumor.   Growth.
Oct. 31, 1924, tumor inoculated with vaccine virus.   Continued tumor growth.
Nov. 28, 1924, *4 weeks later*, tumor removed, emulsified, and inoculated into rabbits as indicated below.
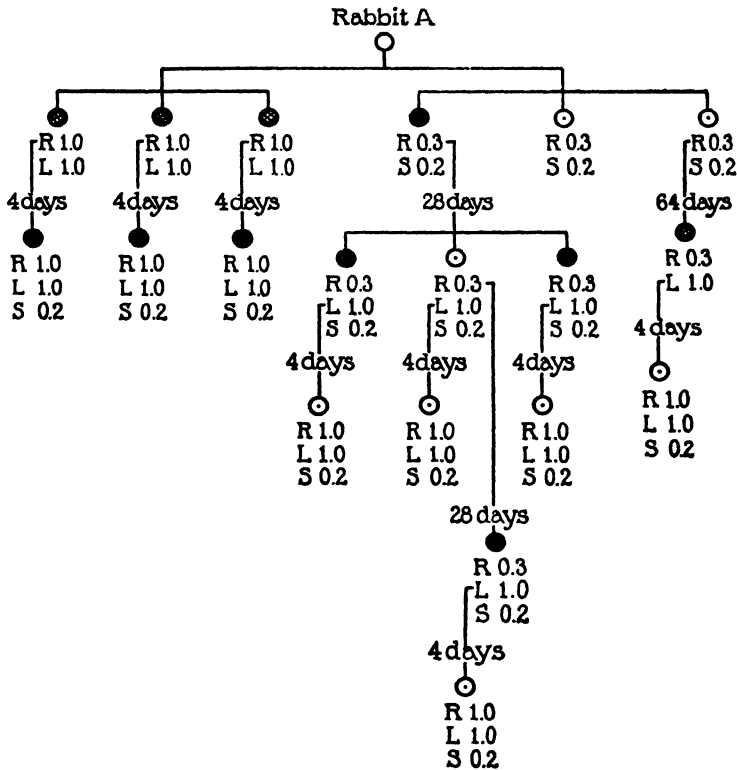
Each circle represents one rabbit.

R indicates right testicle; L, left testicle; S, skin.   Figures following R, L, and S indicate amounts in cc.

Transplants of tumor were made in right testicles at intervals of 28 to 64 days as indicated.

Passages from animal to animal for the demonstration and identification of vaccine virus in the tumor were made as indicated at 4 day intervals by injecting tumor or testicular emulsion into the testicles and skin of rabbits.

● indicates presence of vaccine virus in testicles.   Animal immune to vaccine virus inoculated intradermally 3 weeks later.

⊙ indicates presence of vaccine virus in testicles, vaccine pustules in skin. Animals not tested for immunity.

● indicates presence of vaccine virus in testicles, vaccine pustules in skin. Animals immune to vaccine virus inoculated intradermally 3 weeks later.

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the one time.   Several generations of this strain of tumor have been studied, transfers being made at intervals of 28 to 64 days.   All rabbits inoculated with it become refractory to skin infection with vaccine virus and the presence of vaccine virus in the tumor has been repeatedly demonstrated.   A few of the experiments are graphically illustrated in Text-fig. 4.

Noguchi (4) recovered vaccine virus from experimentally infected testicles of the rabbit 28 days after inoculation.   He was unable to recover it later than this and places the outside limit of survival at 5 weeks.   We have been able to demonstrate the virus in tumors several generations removed from the experimentally infected tumor and it is evident that it multiplies and is carried to successive generations of the tumor.   Since the tumor transplants at times were made at intervals of 64 days it is also apparent that vaccine virus survives longer in the tumor than it does in testicular tissue, despite the fact of the immune state of the rabbit host.   This condition is similar to that encountered in connection with Virus III.

DISCUSSION.

The experiments reported in this paper confirm Levaditi's observations concerning the growth and unusually long survival of vaccine virus in neoplasms.   In addition, we have shown that a filterable virus indigenous to rabbits behaves in a similar manner in a transplantable neoplasm of the same animal species.   We agree with him that malignant cells may not be able to develop an active immunity to viruses and that this may be the explanation for the long survival of the viruses in the rabbit tumor of our experiments.   We feel, however, that a great deal more evidence is needed before this explanation can be accepted as the correct one.   If it be correct, there will remain the further question whether the inability of cells to develop an active immunity is unique with tumor cells.   In any event, it would seem that the use of a transplantable actively growing tissue, such as the rabbit tumor, offers a suitable medium *in vivo* and possibly *in vitro*, for the growth and study of a number of viruses and possibly of other agents of disease.   Such procedures may also lead to observations which will have a significant bearing upon the biological reaction to various infectious agents of tumor cells as contrasted with normal cells.

SUMMARY.

Virus III and vaccine virus multiply in a transplantable rabbit tumor of epithelial origin; are carried along with the tumor through an indefinite number of transplants; and despite an immunity developed by the rabbit host survive longer in the tumor than when injected into the testicles of normal rabbits.

BIBLIOGRAPHY.

1. Levaditi, C., and Nicolau, S., *Ann. Inst. Pasteur*, 1923, xxxvii, 443.
2. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673; 1924, xxxix, 777; xl, 281.
3. Miller, C. P., Jr., Andrewes, C. H., and Swift, H. F., *J. Exp. Med.*, 1924, xl, 773. Andrewes, C. H., and Miller, C. P., Jr., *J. Exp. Med.*, 1924, xl, 789.
4. Noguchi, H., *J. Exp. Med.*, 1915, xxi, 539.
5. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 601, 631, 799, 811; xxxviii, 347. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 583.
6. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 799.
7. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1924, xl, 583

STUDIES OF ACIDOSIS.

XXI. The Colorimetric Determination of the pH of Urine.

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Colorimetric methods for the determination of urine pH have been described by Henderson and Palmer (1), Michaelis (2), and others, which are sufficiently accurate for most clinical purposes. Quagliariello and D'Agostino (3) proposed a colorimetric method, which checked their electrometric determinations with an error ranging from -0.16 to $+0.15$ pH. They employed phosphate buffers as standards, with neutral red and para-nitrophenol indicators. The temperature was not controlled, nor was the dye concentration in the standard and unknown solutions uniform throughout the range of any one indicator. Apparently no attempt was made to prevent loss of CO_2 from urine samples, or to take into consideration the partial pressure of this gas in the electrometric measurements. Marshall (4) has shown that the loss of CO_2 from urine specimens may cause quite an appreciable error in pH determinations, amounting to several tenths of a pH in some alkaline samples.

In the present paper we have attempted to increase the accuracy of the colorimetric method, and have controlled it by comparison with electrometric measurements. Due to reasons which will be pointed out, the accuracy of the method, even with the precautions employed, is only 0.1 pH as compared with an accuracy of ±0.02 pH in the case of blood determinations. The bicolor standards, used by Hastings and Sendroy (5) for blood plasma pH measurements, have been adapted to urine.

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Method.

The urine is collected and kept under oil. 2 cc. are pipetted without exposure to air into a test-tube containing 1 cc. of indicator solution and 7 cc. of redistilled water under oil. As a control tube, another 2 cc. portion is run into a similar test-tube with 8 cc. of water. After gently stirring with a footed rod, the unknown solution is brought to 38° in a water bath and matched with pairs of standard indicator solutions as previously described (5). To allow for the error introduced by dilution of the urine, 0.10 pH is subtracted from the reading at 38°. This corrected pH has been found to be within 0.05 of the electrometric pH at the same temperature.

The apparatus required is an adequate number of clear glass test-tubes (22 × 175 mm.) of uniform inside diameter, a calibrated micro burette graduated to 0.02 cc., a 50 cc. burette, and a 3-row comparator block.

Preparation of the Color Standards.—The color standards used for comparison with the unknown are prepared as outlined in Tables I to IV.

Each pH standard consists of two tubes, one of which contains a known concentration of the indicator in its alkaline form, the other in its acid form. The concentrations are so chosen that the sum of the concentrations of indicator in the two tubes of each pair is constant throughout the series.

This method has been used by Bjerrum, Michaelis, Sörensen, Gillespie, Myers, and others. It is based on the assumption that the color of an indicator solution is due to a mixture of the acid and alkaline forms of the indicator. Each form has its peculiar color, and the proportions of the two forms are related to the reaction of the solution as indicated by the Henderson-Hasselbalch equation for solutions of buffer acids and their salts, *viz.*

$$pH = pK' + \log \frac{BA}{HA}$$

where BA is the alkaline salt, in this case the alkaline form of the indicator, and HA is the free acid, or the acid form of the indicator. Variations in the ratio of the alkaline form to the acid form correspond to variations in pH according to the above equation. Values

for the pK' of the indicators used at room temperature have been determined by Clark (6), Gillespie (7), Brode (8), and Holmes and Snyder (9), and have been verified by the authors. Additional values of pK' at 38° have also been determined by the authors.

The quantities of indicator solution to be added to each tube are accurately measured from a micro burette. To each tube either dilute acid or alkali is then added to make a total volume of 25 cc.

TABLE I.

Table for Preparation of Bicolor Standards, with 0.016 Per Cent Brom Cresol Green, 0.002 N HCl, and 0.001 N NaOH. Brom Cresol Green pK' = 4.72 at 38° and 20°.

pH$_{38°}$ and 20°	Alkali tube.		Acid tube.	
	cc. dye	*cc. alkali*	*cc. dye*	*cc. acid*
4.00	0.40	24.60	2.10	22.90
4.10	0.49	24.51	2.01	22.99
4.20	0.58	24.42	1.92	23.08
4.30	0.69	24.31	1.81	23.19
4.40	0.81	24.19	1.69	23.31
4.50	0.94	24.06	1.56	23.44
4.60	1.08	23.92	1.42	23.58
4.70	1.23	23.77	1.27	23.73
4.80	1.38	23.62	1.12	23.88
4.90	1.51	23.49	0.99	24.01
5.00	1.64	23.36	0.86	24.14
5.10	1.77	23.23	0.73	24.27
5.20	1.88	23.12	0.62	24.38
5.30	1.98	23.02	0.52	24.48
5.40	2.07	22.93	0.43	24.57
5.50	2.14	22.86	0.36	24.64
5.60	2.21	22.79	0.29	24.71
5.70	2.26	22.74	0.24	24.76
5.80	2.31	22.69	0.19	24.81

(Due to its greater stability, 0.001 N HCl has been substituted for the 0.0001 N HCl originally prescribed (5) for the acid tubes of the phenol red series.) The tubes are stoppered or sealed, and kept in a dark cupboard. When sealed, the solutions are stable for several months.

Preparation of Indicator Solutions.—The indicators, brom cresol green, brom cresol purple, and phenol red, covering a pH range of

TABLE II.

Table for Preparation of Bicolor Standards with 0.01 Per Cent Chlor Phenol Red, 0.001 N HCl, and 0.01 N NaOH. Chlor Phenol Red pK' = 5.93 at 38°, and 6.02 at 20°.

pH$_{38°}$	Alkali tube.		Acid tube.		pH$_{20°}$
	cc. dye	cc. alkali	cc. dye	cc. acid	
5.00	0.26	24.74	2.24	22.76	5.09
5.10	0.32	24.68	2.18	22.82	5.19
5.20	0.39	24.61	2.11	22.89	5.29
5.30	0.48	24.52	2.02	22.98	5.39
5.40	0.57	24.43	1.93	23.07	5.49
5.50	0.68	24.32	1.82	23.18	5.59
5.60	0.80	24.20	1.70	23.30	5.69
5.70	0.93	24.07	1.57	23.43	5.79
5.80	1.07	23.93	1.43	23.57	5.89
5.90	1.20	23.80	1.30	23.70	5.99
6.00	1.35	23.65	1.15	23.85	6.09
6.10	1.50	23.50	1.00	24.00	6.19
6.20	1.63	23.37	0.87	24.13	6.29
6.30	1.75	23.25	0.75	24.25	6.39

TABLE III.

Table for Preparation of Bicolor Standards with 0.008 Per Cent Brom Cresol Purple, 0.002 N HCl, and 0.01 N NaOH. Brom Cresol Purple pK' = 6.09 at 38°, and 6.19 at 20°.

pH$_{38°}$	Alkali tube.		Acid tube.		pH$_{20°}$
	cc. dye	cc. alkali	cc. dye	cc. acid	
5.60	0.61	24.39	1.89	23.11	5.70
5.70	0.72	24.28	1.78	23.22	5.80
5.80	0.85	24.15	1.65	23.35	5.90
5.90	0.99	24.01	1.51	23.49	6.00
6.00	1.12	23.88	1.38	23.62	6.10
6.10	1.26	23.74	1.24	23.76	6.20
6.20	1.40	23.60	1.10	23.90	6.30
6.30	1.55	23.45	0.95	24.05	6.40
6.40	1.68	23.32	0.82	24.18	6.50
6.50	1.80	23.20	0.70	24.30	6.60
6.60	1.91	23.09	0.59	24.41	6.70
6.70	2.01	22.99	0.49	24.51	6.80
6.80	2.09	22.91	0.41	24.59	6.90
6.90	2.16	22.84	0.34	24.66	7.00

4.0 to 8.2, have been used. The dissociation curves of these dyes overlap each other within the useful range and they are probably the most suitable for urine work at this time. Brom cresol green, as recommended by Cohen (10), has displaced methyl red. Chlor phenol red may sometimes prove useful, when difficulty is experienced in reading the higher pH standards of brom cresol green. However, believing that the three other indicators fulfill most requirements, we recommend that chlor phenol red be used only when necessary.

TABLE IV.

Table for Preparation of Bicolor Standards with 0.0075 Per Cent Phenol Red, 0.001 N HCl, and 0.01 N NaOH. Phenol Red pK' = 7.65 at 38°, and 7.78 at 20°.

$pH_{38°}$	Alkali tube.		Acid tube.		$pH_{20°}$
	cc. dye	*cc. alkali*	*cc. dye*	*cc. acid*	
6.70	0.25	24.75	2.25	22.75	6.83
6.80	0.31	24.69	2.19	22.81	6.93
6.90	0.38	24.62	2.12	22.88	7.03
7.00	0.46	24.54	2.04	22.96	7.13
7.10	0.55	24.45	1.95	23.05	7.23
7.20	0.65	24.35	1.85	23.15	7.33
7.30	0.77	24.23	1.73	23.27	7.43
7.40	0.90	24.10	1.60	23.40	7.53
7.50	1.04	23.96	1.46	23.54	7.63
7.60	1.18	23.82	1.32	23.68	7.73
7.70	1.32	23.68	1.18	23.82	7.83
7.80	1.46	23.54	1.04	23.96	7.93
7.90	1.60	23.40	0.90	24.10	8.03
8.00	1.73	23.27	0.77	24.23	8.13
8.10	1.85	23.15	0.65	24.35	8.23
8.20	1.95	23.05	0.55	24.45	8.33

Although exhibiting dichromatism, brom cresol purple, in the absence of a better indicator covering the same range, is still indispensable.

Stock solutions of the indicators were made by dissolving 0.1 gm. of dye in 1 or 1.1 equivalents of NaOH, as recommended by Clark. For convenience these directions will be repeated. 0.1 gm. of each dye is ground in an agate mortar with the following quantities of 0.05 N NaOH.

Indicator.	Equivalents of NaOH to add.	0.05 N NaOH solutions to be added per 0.1 gm. of indicator.
		cc.
Phenol red......................................	1.0	5.7
Brom cresol purple...............................	1.1	4.1
Chlor phenol red.................................	1.1	5.2
Brom cresol green...............................	1.1	3.2

After the dye is completely dissolved in the alkali, the solution is transferred to a 100 cc. volumetric flask, and diluted to the mark. From these stock solutions the more dilute concentrations used in the determination of pH are prepared as needed. The latter are prepared by diluting the stock 0.1 per cent solutions as follows:

Indicator.	Final concentrations.	Stock solution diluted to 200 cc.
	per cent	cc.
Phenol red......................................	0.0075	15
Brom cresol purple...............................	0.008	16
Chlor phenol red.................................	0.01	20
Brom cresol green...............................	0.016	32

The above concentrations of dye are those found most convenient to read by Daylite lamp. However, slight variation in the concentration of the dye solution used does not affect the accuracy of the readings, provided the same dye solution is used in both standards and unknowns. Samples of indicators are sometimes found to contain an insoluble impurity. If this is but a small amount, it may be filtered off and disregarded.

EXPERIMENTAL.

Determinations of the pK' of Indicators at 38° and 20°.—As outlined in a previous communication (5), these values were determined by comparison of pairs of bicolor standards in dilute alkali and acid with standard tubes of M/15 phosphate or M/5 acetate mixtures containing dye in the same proportion. These buffer mixtures were prepared from Merck's salts, the acetate being recrystallized. The pH of phosphate and acetate solutions was determined electrometri-

cally at 20°, using as a standard of reference the ϵ of the saturated calomel electrode obtained with 0.1 N HCl, assuming a pH of 1.08 at 20°. A correction of −0.03 was made for the phosphate values at 38° while those obtained for the acetates at 20° were used also at 38°, there being no temperature coefficient according to Michaelis (11). From these determinations an average pK' for each indicator at 20° and at 38° was obtained, from which the theoretical dissociation curves were constructed (Fig. 1).

Tables I to IV give the amounts of dye solutions required for the bicolor standards as calculated from the pK' values determined in the above manner.

The constants obtained and others already in the literature are as follows:

Indicator.	pK'	Temperature.	Method.	Author.
		°C.		
Phenol red.	7.90	Room.	Spectrophotometric.	Brode.
	7.9	"	Colorimetric.	Clark.
	7.7	25–30	"	Gillespie.
	7.76	25–28	"	Wu (12).
	7.77	20	"	Barnett and Barnett (13).
	7.78±0.007	20	"	Hastings and Sendroy.
	7.65±0.009	38	"	" "
Brom cresol purple.	6.30	Room.	Spectrophotometric.	Brode.
	6.3	"	Colorimetric.	Clark.
	6.3	25–30	"	Gillespie.
	6.28	20	"	Barnett and Barnett.
	6.19±0.025	20	"	Authors.
	6.09±0.029	38	"	"
Chlor phenol red.	6.02±0.019	20	Colorimetric.	Authors.
	5.93±0.022	38	"	"
Brom cresol green.	4.68±0.01	27	Spectrophotometric.	Holmes and Snyder.
	4.72±0.012	20 and 38	Colorimetric.	Authors.
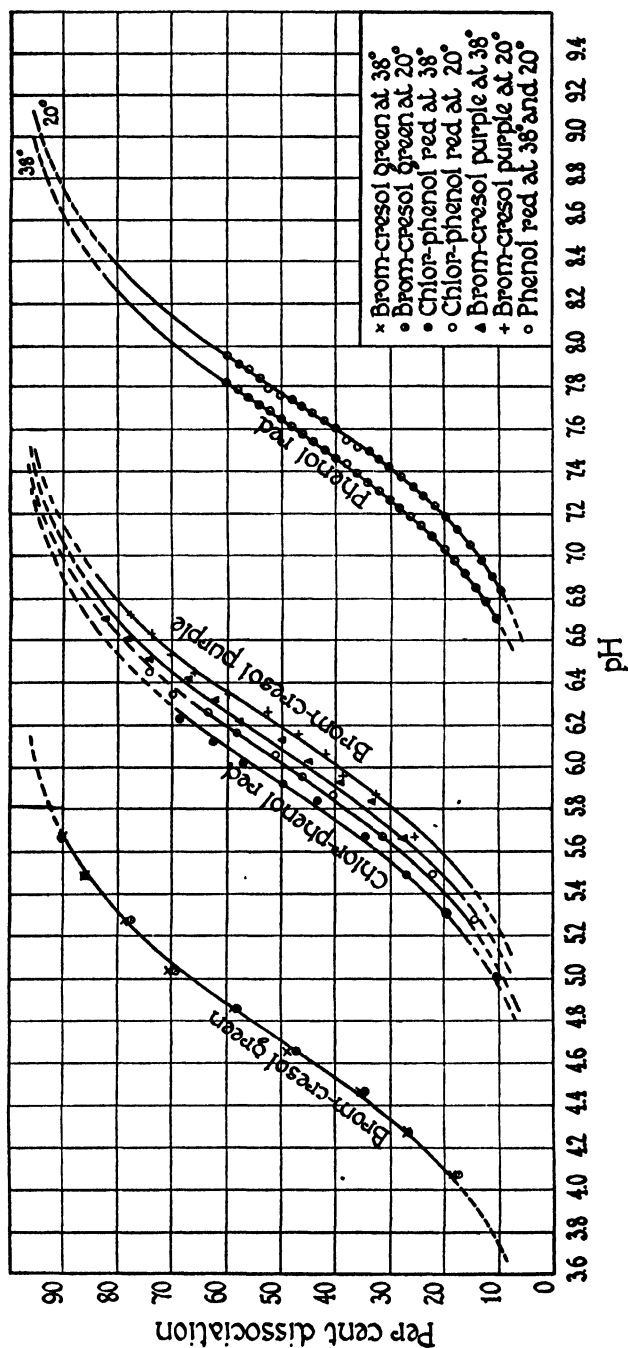
FIG. 1. Calculated and observed dissociation curves for indicators used in urine pH determinations.

While these are only apparent dissociation constants, they hold true for determinations carried out under the prescribed conditions. Due to the optical difficulties encountered in reading the colors of brom cresol purple and chlor phenol red, it has not been found possible to determine these constants with the same order of accuracy as that obtained with the other two. However, inasmuch as individual variations in different lots of dye in themselves may cause deviations comparable to those given above, and since the unknown salt concentration of the urine sample also introduces an error, the values given are thought to be sufficiently close to fall within the limits of error of the method. The dye concentration has been altered from twice to one-half that prescribed above, and in no case has there been any difference from the values given.

The tables given for the preparation of standards are based on the assumption that *the pure dye, of constant pK′ throughout its useful range*, is being used. However, in the experience of other workers (8), individual lots of dye may have different apparent dissociation constants.

Of the several different samples of brom cresol green used in this laboratory, some have been found to be very poor. Samples of brom cresol green in the dry state varied in color from dark purple to an orange reddish tinge, and gave melting points such as 227°, 223°, and 215.5°. The latter, a sample from LaMotte Chemical Products Company, proved to be entirely satisfactory. The dibrom derivative, brom cresol purple, gave a melting point at 243°.

Effect of Dilution on pH of Urine.—Samples of normal urine were saturated twice at 38° with a gas mixture containing CO_2 at 40 mm. tension plus hydrogen to 1 atmosphere. After saturation, urine samples of various dilutions were analyzed electrometrically, with a CO_2 partial pressure corresponding to that of the diluted sample in each case; *e.g.*, if the urine was diluted 5-fold, hydrogen containing CO_2 at 8 mm. tension was used in the electrode vessel. Table V, Sample 1, gives some idea of the magnitude of change of pH taking place on dilution of urine. Samples 2 to 7 were analyzed as delivered under oil or aerated (2 and 3) with CO_2-free hydrogen in the electrode. In these samples the effect of dilution (indicated in Table V as Δ pH) amounted to +0.08 ±0.03 pH. Due to the

TABLE V.

Effect of Dilution on the pH of Urines Determined Electrometrically.

Sample No.	Temperature.	Dilution.	Electrometric pH undiluted.	Electrometric pH diluted.	ΔpH due to dilution.
	°C.				
1	38	0	5.46		
		2×		5.49	+0.03
		3×		5.52	+0.06
		6×		5.56	+0.10
		11×		5.59	+0.13
		16×		5.62	+0.16
2	20	5×	7.14	7.23	+0.09
3		5×	7.81	7.92	+0.11
4		5×	5.58	5.63	+0.05
5		5×	6.22	6.32	+0.10
6		5×	5.54	5.64 ·	+0.10
7		5×	5.64	5.69	+0.05
Average..................		5×	(7 samples.)		+0.08

TABLE VI.

Comparison of pH of Urines at 38° Determined Electrometrically on Undiluted Samples, and Colorimetrically on Samples Diluted Five Times.

Sample No.	Electrometric pH undiluted.	Colorimetric pH diluted five times (uncorrected).	Difference between electrometric and uncorrected colorimetric pH.	Difference between electrometric and corrected colorimetric pH.	Dye used.
1	5.11	5.28	+0.17	+0.07	Brom cresol green.
2	6.03	6.17	+0.14	+0.04	" " purple.
3	6.16	6.31	+0.15	+0.05	" " "
4	6.98	7.07	+0.09	−0.01	Phenol red.
5	6.83	6.92	+0.09	−0.01	" "
6	6.34	6.39	+0.05	−0.05	Brom cresol purple.
7	6.35	6.43	+0.08	−0.02	" " "
8	4.51	4.64	+0.13	+0.03	" " green.
9	4.49	4.58	+0.09	−0.01	" " "
10	5.46	5.60	+0.14	+0.04	Chlor phenol red.
Average.....................			+0.11	±0.03	

difference in nature and amount of salt in individual urine specimens one would not expect a constant correction in all urines at any one dilution, unless it were possible to dilute the urine with an iso-ionic solution. However, one may subtract a correction of 0.1 pH for the effect of 5- to 10-fold dilution without introducing a significant error.

Comparison of Electrometric and Colorimetric Determinations of Urine pH.—Table VI gives electrometric and colorimetric determinations on urines diluted 5-fold after saturation with CO_2 and hydrogen as above. At this dilution, the correction involved in the colorimetric determination may be attributed almost entirely to the effect due to dilution. As one dilutes, the salt error of the dye decreases while the dilution error increases. At 5- and 10-fold dilution, the dilution error accounts for most of the total correction. The next to the last column gives corrected differences between the actual pH and the colorimetric determination. Although urines vary in their content of salts and other substances affecting the dye, and in their behavior on dilution, one may apparently subtract a correction of 0.1 pH from the colorimetrically observed pH in the 5- to 10-fold diluted urine at 38°, and be within 0.1 of the actual pH.

SUMMARY.

The apparent dissociation constants of brom cresol green, chlor phenol red, and brom cresol purple have been determined by the colorimetric method at 38° and 20°.

Using bicolor standards and an empirical correction for the error introduced by dilution of the urine, a technique for the colorimetric determination of urine pH is described.

A comparison of electrometric and colorimetric pH determinations in urine has been made.

BIBLIOGRAPHY.

1. Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.
2. Michaelis, L., *Deutsch. med. Woch.*, 1921, xlvii, 465.
3. Quagliariello, G., and D'Agostino, E., *Deutsch. med. Woch.*, 1912, xxxviii, 2171.
4. Marshall, E. K., Jr., *J. Biol. Chem.*, 1922, li, 3.

5. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1924, lxi, 695.
6. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.
7. Gillespie, L. J., *J. Am. Chem. Soc.*, 1920, xlii, 742.
8. Brode, W. R., *J. Am. Chem. Soc.*, 1924, xlvi, 581.
9. Holmes, W. C., and Snyder, E. F., *J. Am. Chem. Soc.*, 1925, xlvii, 226.
10. Cohen, B., *Bull. Hyg. Lab.*, *U. S. P. H.*, 1923, xxxviii, 199.
11. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition revised, 1922.
12. Wu, H., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 111.
13. Barnett, G. D., and Barnett, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 127.

THE EFFECT OF VARIATION IN IONIC STRENGTH ON THE APPARENT FIRST AND SECOND DISSOCIATION CONSTANTS OF CARBONIC ACID.

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The biological and chemical importance of carbonic acid has stimulated much work upon its first dissociation constant with the immediate object of using this constant for the calculation of the hydrogen ion concentration of biological solutions. Fewer investigations have had as their object the determination of the second dissociation constant.

The mathematical treatment of the behavior of solutions of strong electrolytes by Milner (1) and by Debye and Hückel (2) has brought to the empirical results obtained by Lewis (3) and his collaborators, and Brönsted and La Mer (4) a theoretical explanation which seems adequate. Experimental work has indicated that the deviation in the behavior of strong electrolytes in moderately dilute solution from their behavior in infinitely dilute solution can be approximately expressed in the following manner.

If α = the activity
γ = "   "   coefficient, and
c = " molal concentration of an ion, then
$\alpha = \gamma c$. Further, that the activity coefficient is related to the ionic concentration of the solution by the empirical formula

$$- \log \gamma = \beta v^2 \sqrt{\mu} \text{ where}$$

β = an empirically determined constant which has a value of approximately 0.50
v = the valence and
μ = the ionic strength and is defined as $\frac{1}{2} \Sigma c v^2$.

The theoretical considerations of Debye and Hückel led to an expression which, under certain limiting conditions, corresponded well with the above empirical equation.

In view of these developments and the desirability of knowing

the activity of bicarbonate and carbonate ions for subsequent studies, we have endeavored to determine the effect of varying ionic strength on the apparent dissociation constants of carbonic acid.

FIRST DISSOCIATION CONSTANT.

The first dissociation constant of carbonic acid has been accurately determined by numerous investigators. Of the determinations based on conductivity measurements those of Walker and Cormack (5) and of Kendall (6) are probably the most reliable. The results of these workers, recalculated by Warburg (7), give the value for K_1 as 3.11×10^{-7} at $18°$; $pK_1 = 6.507$. Kendall's value for pK_1 is 6.656 at $0°$ and 6.460 at $25°$. This represents a change in pK_1 per degree of 0.0078. Assuming that this temperature coefficient is valid up to $38°$, the pK_1 of carbonic acid would be 6.36 at body temperature. Hasselbalch (8) in 1916, determined pK_1' electrometrically in bicarbonate solutions ranging in concentration from 0.005 to 0.05 N. The method for measuring electrometric pH in CO_2-containing solutions at that time contained certain errors, which Warburg has pointed out in recalculating Hasselbalch's values for pK_1'. These values are consistently about 0.09 lower than Warburg's and about the same amount lower than ours. The reason for the discrepancy apparently lies in the differences in technique employed in the measurement of pH values. The most accurate determinations of the first dissociation constant by electrometric measurement appear to be those of Warburg. These results we have recalculated, using the solubility coefficient for CO_2 found in this laboratory, and have included in Fig. 1 with our own results.

The mass law equation for the ionization of carbonic acid in terms of activity is

$$\frac{\alpha_{H^{\cdot}} \times \alpha_{HCO_3'}}{\alpha_{H_2CO_3}} = K_1$$

In the logarithmic form this becomes

(1) $$\log \alpha_{H^{\cdot}} + \log \alpha_{HCO_3'} - \log \alpha_{H_2CO_3} = \log K_1$$

where α represents the activity of each individual component. For $\alpha_{HCO_3'}$ one may write $\gamma_1 [HCO_3']$ where γ_1 denotes the activity coeffi-

cient of the HCO_3' ion. Furthermore, in the presence of a relatively large amount of $NaHCO_3$ one may write γ_1 [$NaHCO_3$] instead of γ_1 [HCO_3'], assuming for the moment, that $NaHCO_3$ behaves as a strong electrolyte. Since the activity of a gas is proportional to its tension, one may write

$$\alpha_{H_2CO_3} = \frac{p_{CO_2}}{760} \times \frac{a_{CO_2}}{0.0224}$$
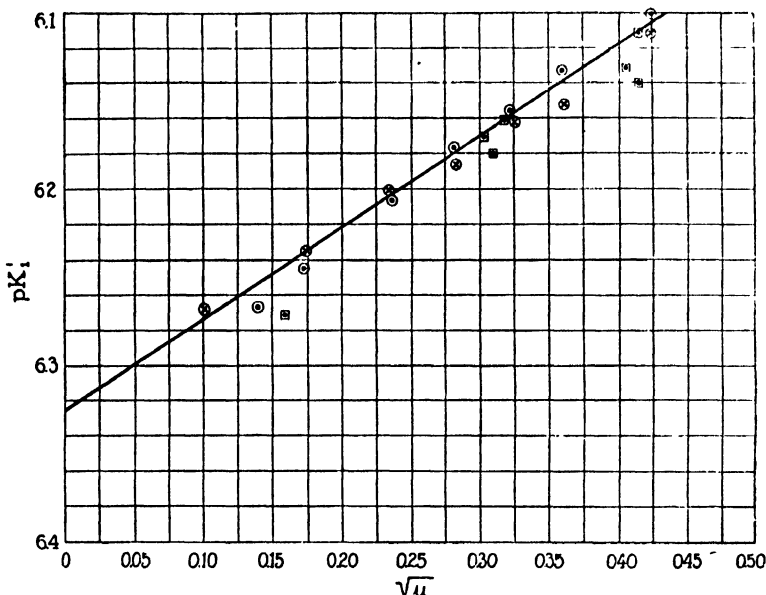
FIG. 1. Values of pK_1' are plotted as ordinates and of $\sqrt{\mu}$ as abscissæ. Points marked ⊙ and ⊗ are from data given in the present paper. Those marked ⊠ indicate data recalculated from Warburg's experiments. The line represents values of pK_1' calculated as $pK_1' = 6.33 - 0.5 \sqrt{\mu}$.

where p_{CO_2} denotes the tension of CO_2 with which the solution is in equilibrium and a_{CO_2} the solubility of CO_2 in the solution at that temperature. In conformity with the recent recommendations of Sörensen and Linderström-Lang (9) we shall write $p\alpha_H$ for $-\log \alpha_H$. With the above substitutions Equation 1 then becomes

(2) $\quad - p\alpha_H + \log [NaHCO_3] - \log p_{CO_2} - \log \dfrac{a_{CO_2}}{760 \times 0.0224}$

$\quad\quad = \log K_1 - \log \gamma_1$

(3) $\quad\quad$ We shall let $pK_1' = -\log K_1' = pK_1 + \log \gamma_1$

Our experiments have been performed with the object of deter-
mining the relation between pK_1' (and consequently log γ_1) and
the ionic strength of the solution.

EXPERIMENTAL.

The experiments to be reported here represent the results of the
determination of pK_1' in solutions of eight different ionic strengths.
Each of these experiments was repeated with a freshly prepared
solution.

The sixteen different solutions whose compositions are given in
Table I were prepared from $NaHCO_3$ and NaCl of a high degree of
purity. Each was rotated for 30 minutes in a water bath at 38°
with hydrogen and CO_2 at a tension previously calculated to give
the desired pH. The saturation was repeated for a second 30 min-
ute period in order to insure equilibrium. The liquid phases were
then separated by the technique described in a previous paper (10)
and the following analyses were made.

Samples of the gas phase were transferred to the Haldane-Henderson
gas apparatus and the tension of CO_2 was determined. The liquid
phase, which was contained in filled glass bulbs over mercury, was
analyzed for its CO_2 content in the Van Slyke (11) manometric gas
apparatus. The $p\alpha_H$ was determined electrometrically at 38° in the
Clark electrode vessel using the cell

Hg	HgCl	Saturated KCl	Saturated KCl bridge	Unknown solution	H₂	Pt

The potential of the cell, with 0.1 N HCl in place of the unknown
solution, was determined at the beginning of each day's experiment.
This value of "ϵ_0", calculated on the assumption that the $p\alpha_H$ of
0.1 N HCl is 1.08, is given in each table. As pointed out by Cullen
(12) this procedure serves as a daily calibration of the cell system
used. The value 1.08, used as the $p\alpha_H$ of 0.1 N HCl, is taken from
Lewis' value for the activity of H· in an HCl solution whose ionic
strength is 0.1 μ.[1]

[1] Lewis and Randall (3), p. 382.

Values of $p\alpha_H$ are calculated from the equation

$$p\alpha_H = \frac{\text{E.M.F. (corrected to 1 atmosphere dry } H_2) - \epsilon_0}{0.06164}$$

TABLE I.

The pK_1' of Carbonic Acid at 38° in Salt Solutions of Varying Ionic Strength.

No.	p_{CO_2}	Solubility coefficient of CO2	H_2CO_3	CO_2	$NaHCO_3$	$\log \frac{NaHCO_3}{H_2CO_3}$	$p\alpha_H$	pK_1'	NaCl	μ	$\sqrt{\mu}$
	mm.		*mM per l.*	*mM per l.*	*mM per l.*				*mM per l.*		
1	61.6	0.552	1.997	12.25	10.25	0.710	6.977	6.267	0.00	0.01025	0.1012
2	65.6	0.552	2.127	12.40	10.27	0.684	6.950	6.266	0.00	0.01027	0.1013
3	117.6	0.550	3.80	23.97	20.17	0.725	6.990	6.265	0.00	0.02017	0.1418
4*	105.4	0.550	3.407	24.12	20.71	0.784	6.960	6.176	0.00	0.02071	0.1439
5	178.6	0.549	5.76	35.56	29.80	0.714	6.957	6.243	0.00	0.02980	0.1726
6	172.8	0.549	5.57	35.62	30.05	0.732	6.967	6.235	0.00	0 03005	0.1734
7	158.1	0.546	5.07	35.10	30.03	0.772	6.976	6.204	24.83	0.05486	0.2342
8	158.5	0.546	5.083	34.96	29.88	0.768	6.970	6.202	24.83	0.05471	0.2343
9	152.3	0.544	4.87	35.12	30.25	0.793	6.970	6.177	49.65	0.07990	0.2827
10	152.4	0.544	4.87	34.86	29.99	0.789	6.975	6.186	49.65	0.07964	0.2822
11	145.0	0.541	4.61	34.75	30.14	0.815	6.970	6.155	74.43	0.10457	0.3234
12	150.3	0.541	4.775	34.90	30.12	0.800	6.962	6.162	74.43	0.10455	0.3233
13	140.5	0.538	4.44	34.86	30.42	0.836	6.970	6.134	99.34	0.12976	0.3602
·14	140.1	0.538	4.427	34.46	30.03	0.831	6.984	6.153	99.34	0.12937	0.3597
15	136.8	0.532	4.28	34.60	30.32	0.850	6.950	6.100	149.00	0.17932	0.4235
16	131.9	0.532	4.122	34.70	30.58	0.870	6.980	6.110	149.00	0.17958	0.4238

In odd numbered experiments $\epsilon_0 = 0.2364$; in even numbered experiments $\epsilon_0 = 0.2351$.

* Loss of CO_2 from gas phase. Not plotted in Fig. 1.

In Table I are given the results of our analyses. The H_2CO_3 is calculated from the CO_2 tension. This is subtracted from the total CO_2 leading to the values for $NaHCO_3$.

In the ninth column are given the values of pK_1' calculated from these data by means of Equation 2. The results of our determinations, together with those found by Warburg, are given in Fig. 1, in which the values pK_1' are plotted as ordinates and the $\sqrt{\mu}$ as abscissæ.

It will be seen that most of the points lie on a straight line which intersects the ordinate at 6.33 and has a slope of 0.5. The equation of this line is therefore

$$pK_1' = 6.33 - 0.5 \sqrt{\mu}$$

Combining Equation 3 with this and substituting 6.33 for pK_1 we obtain

$$-\log \gamma_1 = 0.5 \sqrt{\mu}$$

This empirical equation apparently fits the experimental data even when the ionic strength of the solution is as great as 0.180. Such correspondence would not have been expected from the theory of Debye and Hückel because it is known that in solutions whose ionic concentration is greater than 0.10 μ, correction should be made for the dimensions of the ions present and for the altered dielectric properties of the solvent. This would cause the theoretical line to bend toward the abscissa and our actual points lie slightly above such a curve. It is shown clearly, however, that the curve relating $-\log\gamma_1$ and $\sqrt{\mu}$ is linear and has a slope of the order of magnitude predicted by the Debye and Hückel theory in the region of moderately dilute solutions.

SECOND DISSOCIATION CONSTANT.

Determinations of the second dissociation constant of carbonic acid have been made by McCoy (13), Seyler and Lloyd (14), Bjerrum and Gjaldbaek (15), and others. McCoy's results, as recalculated by Stieglitz (16), led to a value of $K_2 = 5.4 \times 10^{-11}$ or $pK_2 = 10.27$ at 25°; Seyler and Lloyd found $K_2 = 4.3 \times 10^{-11}$ or $pK_2 = 10.37$; and Bjerrum and Gjaldbaek give $K_2 = 6.0 \times 10^{-11}$ or $pK_2 = 10.22$ at 25°. Although it was recognized by McCoy that the concentration of the solution in which the constant was determined affected the constant obtained, no systematic study of the effect of varying ionic strength on the second dissociation constant has been made.

As in the case of the first dissociation constant we are here concerned with the mass law equation,

$$(4) \qquad \frac{\alpha_H \cdot \times \alpha_{CO_3''}}{\alpha_{HCO_3'}} = K_2$$

which in the logarithmic form becomes

$$(5) \qquad \log \alpha_H \cdot + \log \alpha_{CO_3''} - \log \alpha_{HCO_3'} = \log K_2$$

Substituting $\gamma_2 [CO_3'']$ and $\gamma_1 [HCO_3']$ for $\alpha_{CO_3''}$ and $\alpha_{HCO_3'}$ respectively, Equation 5 may be written

$$(6) \qquad \log \alpha_H \cdot + \log \gamma_2 [CO_3''] - \log \gamma_1 [HCO_3'] = \log K_2$$

We have shown in the previous section that if $[HCO_3']$ be taken equal to $[NaHCO_3]$ then $-\log \gamma_1 = 0.5 \sqrt{\mu}$. By making the assumption that Na_2CO_3 also is a strong electrolyte, so that $[CO_3''] = [Na_2CO_3]$, Equation 6 may be rewritten

$$(7) \quad \log \alpha_H \cdot + \log [Na_2CO_3] + \log \gamma_2 - \log [NaHCO_3] + 0.5 \sqrt{\mu} = \log K_2$$

Transposing $\log \gamma_2$ and $0.5\sqrt{\mu}$ to the right-hand side, dividing through by minus one and substituting $p\alpha_H$ for $-\log \alpha_H$, Equation 7 becomes

$$(8) \quad p\alpha_H - \log [Na_2CO_3] + \log [NaHCO_3] = -\log K_2 + \log \gamma_2 + 0.5 \sqrt{\mu} = pK_2'$$

Our experimental work has had for its purpose the determination of pK_2' in solutions of different ionic strengths. From these results we have been able to determine the relation between $\log \gamma_2$ and the ionic strength.

EXPERIMENTAL.

As in the case of the first dissociation constant, sixteen different solutions were prepared, representing eight different ionic strengths. The CO_2 of each solution was determined gasometrically and the total alkali ($NaOH + Na_2CO_3 + NaHCO_3$) titrimetrically. Using Michaelis' (17) value of 13.475 for pK_w at 38°, $p\alpha_{OH'}$ was obtained. From the activity coefficient of OH' in solutions of different ionic

strength, as given in Lewis and Randall, the NaOH concentration was calculated.

From the ratio of Na_2CO_3 + $NaHCO_3$ to total CO_2 the Na_2CO_3 and $NaHCO_3$ were calculated. A ratio of Na_2CO_3 to $NaHCO_3$ was

TABLE II.

The pK_2' of Carbonic Acid at 38° in Salt Solutions of Varying Ionic Strength.

No.	Na as NaOH, Na₂CO₃, and NaHCO₃.	NaCl	NaOH	Na₂CO₃ + NaHCO₃.	Total CO₂ as (Na₂CO₃+ NaHCO₃).	Na₂CO₃	NaHCO₃	$\log \frac{Na_2CO_3}{HCO_3}$	p_{α_H}	pK_2'	μ	$\sqrt{\mu}$
	m.-Eq. per l.	mM per l.	mM per l.	m.-Eq. per l.	mM per l.	mM per l.	mM per l.					
1	15.35	0.00	0.40	14.95	10.07	4.88	5.19	−0.027	10.026	10.053	0.02023	0.1422
2	15.35	0.00	0.40	14.95	10.02	4.93	5.09	−0.014	10.027	10.041	0.02028	0.1424
3	30.10	0.00	0.39	29.71	19.76	9.95	9.81	0.006	10.005	9.999	0.04005	0.2001
4	30.10	0.00	0.40	29.70	19.78	9.92	9.86	0.003	10.010	10.007	0.04002	0.2000
5	45.13	0.00	0.37	44.76	29.59	15.17	14.42	0.022	9.969	9.947	0.06030	0.2455
6	45.20	0.00	0.38	44.82	29.66	15.16	14.50	0.019	9.979	9.960	0.06036	0.2457
7	45.20	24.83	0.34	44.86	29.56	15.30	14.26	0.031	9.914	9.883	0.08535	0.2922
8	45.20	24.83	0.35	44.85	29.57	15.28	14.29	0.030	9.932	9.902	0.08532	0.2921
9	45.27	49.65	0.31	44.96	29.65	15.31	14.34	0.029	9.867	9.838	0.11025	0.3320
10	45.20	49.65	0.31	44.89	29.47	15.42	14.05	0.041	9.872	9.831	0.11029	0.3321
11	45.13	74.43	0.29	44.84	29.80	15.04	14.76	0.008	9.836	9.828	0.13462	0.3669
12	45.27	74.43	0.30	44.97	29.54	15.43	14.11	0.039	9.846	9.807	0.13515	0.3676
13	45.20	99.34	0.27	44.93	29.67	15.26	14.41	0.025	9.797	9.772	0.15983	0.3998
14	45.20	99.34	0.27	44.93	29.45	15.48	13.97	0.045	9.797	9.752	0.16005	0.4001
15	45.27	149.00	0.25	45.02	29.64	15.38	14.26	0.033	9.753	9.720	0.20968	0.4579
16	45.20	149.00	0.25	44.95	29.58	15.37	14.21	0.034	9.752	9.718	0.20960	0.4578

In odd numbered experiments ϵ_0 = 0.2350; in even numbered experiments ϵ_0 = 0.2349.

chosen which gave the maximum accuracy and was sufficiently high so that the H_2CO_3 concentration could be neglected. The $p\alpha^H$ of each solution was determined electrometrically using as ϵ_0 the values found with 0.1 N HCl at the beginning of each experiment.

From these data and Equation 8, we have calculated pK_2' for each solution. Our results are tabulated in Table II and graphically represented in Fig. 2 with pK_2' as ordinates and $\sqrt{\mu}$ as abscissæ. The equation which satisfies this line is

$$pK_2' = 10.22 - 1.1 \sqrt{\mu}$$
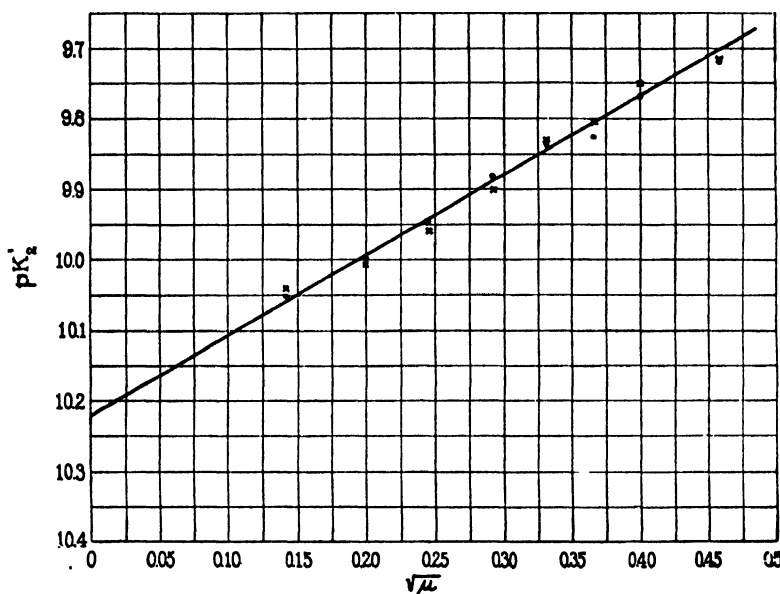
FIG. 2. Values of pK_2' are plotted as ordinates and of $\sqrt{\mu}$ as abscissæ. The line represents values of pK_2' calculated as $pK_2' = 10.22 - 1.1 \sqrt{\mu}$. The points represent experimental results.

Combining with this Equation 8, and putting $pK_2 = 10.22$, we obtain

$$- \log \gamma_2 = 1.6 \sqrt{\mu}$$

If the simple relationship $- \log \gamma = 0.5\ v^2 \sqrt{\mu}$ held for the carbonate ion one might expect the relation between $- \log \gamma_2$ and the $\sqrt{\mu}$ to be

$$- \log \gamma_2 = 0.5 \times 2^2 \sqrt{\mu} = 2 \sqrt{\mu}$$

It is seen that the slope over the range of concentrations with which we worked is somewhat less than that expected from the theory. In view of the fact that no account has been taken of the

dimensions of the ions, or of the change in dielectric constant of the solution at the higher concentrations, it is felt that the correspondence of our results with what might have been anticipated from theoretical considerations is satisfactory. As in the case of γ_1, the linear relationship between $-\log \gamma_2$ and $\sqrt{\mu}$ appears to hold to a concentration of $\mu = 0.16$.

<div align="center">CONCLUSIONS.</div>

The first and second dissociation constants of carbonic acid have been determined at 38° in solutions of varying ionic strength.

When extrapolated to $\mu = 0.0$, the first dissociation constant was found to be 4.68×10^{-7} or $pK_1 = 6.33$. The activity coefficient of the bicarbonate ion, γ_1, was found to be related to the ionic strength of the solution from $\mu = 0.01$ to $\mu = 0.18$ according to the equation

$$- \log \gamma_1 = 0.5 \sqrt{\mu}$$

The apparent first dissociation constant is related to the ionic strength of the solution according to the equation

$$pK_1' = 6.33 - 0.5 \sqrt{\mu}$$

The second dissociation constant of carbonic acid was found to be 6.03×10^{-11} or $pK_2 = 10.22$ at 38°. The activity coefficient of the carbonate ion, γ_2, was found to be related to the ionic strength of the solution from $\mu = 0.02$ to $\mu = 0.16$ according to the equation

$$- \log \gamma_2 = 1.6 \sqrt{\mu}$$

The apparent second dissociation constant is related to the ionic strength of the solution according to the equation

$$pK_2' = 10.22 - 1.1 \sqrt{\mu}$$

These results are in harmony with the theory of the behavior of strong electrolytes in dilute solutions as elaborated by G. N. Lewis, Brönsted, and Debye and Hückel.

<div align="center">BIBLIOGRAPHY.</div>

1. Milner, S. R., *Phil. Mag.*, 1912, xxiii, series 6, 551.
2. Debye, P., and Hückel, E., *Physik. Z.*, 1923, xxiv, 185.

3. Lewis, G. N., and Randall, M., Thermodynamics, New York and London, 1923.
4. Brönsted, J. N., and La Mer, V. K., *J. Am. Chem. Soc.*, 1924, xlvi, 555.
5. Walker, J., and Cormack, W., *J. Chem. Soc.*, 1900, lxxvii, 5.
6. Kendall, J., *J. Am. Chem. Soc.*, 1916, xxxviii, 1480.
7. Warburg, E. J., *Biochem. J.*, 1922, xvi, 153.
8. Hasselbalch, K. A., *Biochem. Z.*, 1916–17, lxxviii, 119.
9. Sörensen, S. P. L., and Linderström-Lang, K., *Compt. rend. Lab. Carlsberg*, 1924, xv, No. 6.
10. Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.
11. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.
12. Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.
13. McCoy, H. N., *Am. Chem. J.*, 1903, xxix, 437.
14. Seyler, C. A., and Lloyd, P. V., *J. Chem. Soc.*, 1917, cxi, 994.
15. Bjerrum, N., and Gjaldbaek, J. K., *Kong. Vet.-og Land.-bohöjskole Aarskr.*, 1919, xlviii, 91.
16. Stieglitz, J., in Chamberlain, T. C., et al., *Carnegie Institution of Washington, Pub. No. 107*, 1909, 234.
17. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition revised, 1922.

THE USE OF UREA AS A DIURETIC IN ADVANCED HEART FAILURE.

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It has been known for a long time that the administration of urea in large doses produces a considerable increase in the volume of urine. Urea was, however, until comparatively recently little utilized for its diuretic properties. This was doubtless due to the prevailing idea that urea retention was the causative factor in uremia. Urea was considered a harmful substance and any measures undertaken to raise its concentration in the body were regarded with disfavor. With the advance of clinical chemistry it soon came to be recognized that although the urea in the blood is raised in uremia it is not the cause of the condition. It became established that if the function of the kidney is good urea can be given to patients for prolonged periods of time with impunity. Even in cases in which kidney disease was known to be present, large doses of urea have been given in order to estimate the functional capacity of the kidney and no untoward results have been observed. Recently, urea has been administered over prolonged periods of time in the treatment of certain cases of nephritic edema in which the blood urea was not raised and very favorable results have been reported.

Urea was first used as a diuretic by Friedrich[1] in 1892. In this investigation he administered urea in edema due to various causes; the series included a few cases of cardiac edema. He gave from 2 to 14 gm. a day and reported very favorable results. Feilchenfeld[2] conducted a similar study with similar results. He stated that the increase in urine volume was proportional to the dose. Strauss[3] gave

1. Friedrich, W.: (Ueber die harntreibende Eigenshaft des Uream) as ureum hugyhajto tulajdonsargarol. Kozleminy u budapeste Magy. kir tud Egyetem gyogyozertani intezelebol, Magy. orv. Arch. 1:400–415, 1892.

2. Feilchenfeld, J.: Ueber Harnstoff als Diureticum, Therap. d. Gegenw. 59: 273, 1918.

3. Strauss, H.: Ueber Harnstoff als Diuretikum, 58:375, 1921.

urea in doses up to 100 gm. a day to twelve patients with cardiac edema following heart disease or arteriosclerosis, with very beneficial results. He commenced with doses of 40 gm. a day and gradually increased it as necessity demanded. In spite of the favorable results reported by the foregoing observers, urea has not come into common use as a diuretic in cardiac edema for reasons we have not been able to ascertain.

For our investigation we have selected cases of advanced cardiac decompensation which have responded only partially or not at all to the methods usually employed in the treatment of cardiac edema. The series included four cases of mitral stenosis with auricular fibrillation, two cases of mitral stenosis, one case of aortic disease with auricular fibrillation and one case of exophthalmic goiter with auricular fibril-lation.

The routine treatment of all patients included rest in bed, a salt free diet, thorough digitalization and restriction of the fluid intake to 1,200 cc. a day. Four of the patients had also received novasurol, and had been rendered edema free, but after the injections of this drug had been discontinued, the urine volume was subnormal and edema had gradually reappeared. Urea was given with a view to reestablish-ing a normal water balance by maintaining an adequate urine output, and also, when slight edema had collected, to bring about its removal. The other four patients of our series all exhibited marked edema when urea medication was instituted. These patients had not received a course of novasurol. One case of mitral stenosis with regular rhythm (Case 6) did not receive digitalis as we decided to study the effect of urea without previous digitalization.

In all cases twelve hour specimens of urine were collected, and in these, the volume, the specific gravity and the chlorid excretion were determined. Urea administration was not commenced until the urine volume and chlorid excretion had reached a constant level. In order to study in detail the changes that took place in the urine volume, urea excretion and blood urea, and their time relations, we collected a speci-men of urine over a period of seventy-two minutes at frequent intervals and obtained a specimen of blood in the middle of this period. The time of making these tests was varied with relation to the administra-tion of urea in order that its effect might be followed more closely.

During these periods no food or fluid was given. The urea in the blood and urine was estimated by the method of Van Slyke and Cullen,[4] the chlorid in the urine by a Volhard titration and the chlorid in the plasma by the method of Van Slyke.[5]

Method of Administration.

Urea was given in a small quantity of water a short time after a meal as it had been previously found that gastric disturbance was less likely to take place if it were given at this time. Urea has a peculiar metallic taste and is not pleasant to take. The patients soon become accustomed to it, however. In four of our cases we administered 30 gm. a day in divided doses of 15 gm. One dose was given at 8:30 a.m. and the other at 4 p.m. In the other four cases, we studied the effect of increasing the quantity of urea. At first 30 gm. a day was given. This was then increased to 45 gm. a day, and in two cases it was further increased to 60 gm. a day. In three of the cases these increases took place at weekly intervals. The urea was given in three equal doses at 8:30 a.m., 12:30 and 6 p.m.

Observations.

Urine Volume.—The response of the cases with edema to urea feeding was prompt and, as a rule, very efficient (accompanying chart). In seven cases of the series, oliguria was present before treatment was instituted. Their average daily output during the control period varied from 340 to 776 cc. After 30 gm. of urea a day, the average daily urine volume for each case varied, as a rule, between 900 and 1,300 cc. When the amount was increased to 45 gm. a day, the average daily output increased to 1,400 and 1,500 cc. A dose of 60 gm. daily was followed by a further slight increase (Tables 1, 2 and 3). The maximum response to a particular dose did not occur until the second or even the third day that the urea was given. The daily output then remained at this level as long as this particular dosage was

4. Van Slyke, D. D., and Cullen, G. E.: A Permanent Preparation of Urease and Its Use in the Determination of Urea, J. Biol. Chem. 19:211, 1914.
5. Van Slyke, D. D.: The Determination of Chlorids in Blood and Tissues. J. Biol. Chem. 58:523 (Dec.) 1923.

continued. After urea administration was discontinued, a diuretic action was still distinguishable during the first twenty-four hour period thereafter.
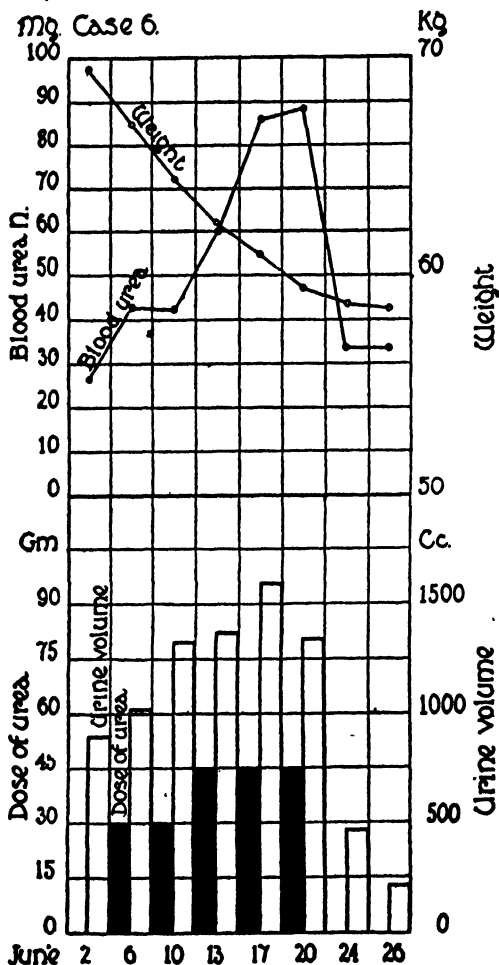
Effect of urea administration on urine volume, weight and blood urea. The patient had received one-third the daily dose of urea at 8:30 and 12:30; specimens of blood were obtained at 2 p.m.

The time relations between the dosage and the diuretic response can be still further analyzed by studying the rate of excretion during the seventy-two minute periods. From these data it may be seen (Table 2)

that an increased urine volume was already established within three hours after the first dose was given. After the urea feeding had been discontinued, the duration of its diuretic action depended to some extent on the dosage. When 30 gm. a day was given, its effect had practically passed off within fifteen to seventeen hours. But with larger doses, its influence was still apparent at this time (Table 1).

Weight of Patient.—Patients with edema lost weight corresponding to the diuresis that took place. This loss of weight was gradual and continuous (chart). Patient 7, who did not have oliguria during the control period, is particularly noteworthy. In this case the diuresis was much greater than in any of the others and the weight loss much more rapid, amounting to 8 kg. in the first five days; this is to be contrasted with a loss of 1.6 kg. in the preceding control period of five days. In some cases continuance of urea feeding was necessary to prevent accumulation of edema, for when it was stopped the patient's weight tended to increase, until visible edema reappeared (Tables 1, 2 and 3).

Clinical Condition.—Corresponding to the weight loss, one noted a disappearance of edema, and of transudates from the body cavities. It was possible to clear all the patients of excess fluid in this way, with the exception of Patient 8. At the same time definite subjective improvement occurred. The most striking clinical improvement occurred in Case 7, in which rapid subjective improvement occurred simultaneously with the loss of edema. Patients 4, 5 and 6 also experienced very definite symptomatic relief which other measures had failed to give. This relief was associated with a decrease in weight. Cases 1 and 2 were free from edema at the beginning of treatment, and there was no definite symptomatic improvement due to urea. Case 3 improved subjectively when urea medication was commenced, although edema was absent and weight loss did not occur. The value of urea in the last three cases was in maintaining a normal water balance. When the urine volume diminished consequent on the withdrawal of urea, edema tended to recur, with its concomitant symptoms. When the administration of urea was resumed, symptoms and edema were again controlled.

In Case 8, urea administration did not result in clinical improvement.

This was a case of mitral stenosis with normal rhythm in which there was considerable ascites, and slight edema of the legs. The patient reacted to a slight extent to digitalis, but this effect was transitory. The dose of urea was gradually increased up to 60 gm. a day, each increase causing a definite rise in urine volume. Nevertheless, the clinical condition did not improve. After nineteen days of treatment the patient had lost 4.5 kg. in weight. The patient began to complain of nausea and on the last day of the course vomiting occurred. The blood urea nitrogen was high after the midday dose, 74.9 mg. per hundred cubic centimeters. Accordingly, urea was discontinued. The urine volume immediately fell and the general state of the patient became worse. It then became apparent that urea had been beneficial to the extent of postponing the unfavorable clinical course that subsequently developed.

Evidence of Intolerance.—When the dose was taken on an empty stomach, vomiting occasionally occurred immediately after, but this symptom gave little trouble when the urea was given after meals. In three of the cases (Cases 5, 7 and 8) the prolonged administration of urea in large doses gave rise to untoward symptoms. These manifestations consisted in loss of appetite, nausea and sometimes vomiting, and in one instance, a feeling of weakness and lassitude. In Cases 7 and 8 they were sufficiently urgent to call for the discontinuance of urea administration. In these cases the blood urea nitrogen, estimated in the afternoon after the second daily dose, was 89.0 mg. and 74.9 mg. per hundred cubic centimeters, respectively. These figures are practically identical with those found by Hewlett, Gilbert and Wickett[6] in experiments designed to investigate the toxicity of urea. In normal subjects they found that similar symptoms occurred when the blood urea reached values above 150 mg. per hundred cubic centimeters (equivalent to 70 mg. of urea nitrogen per hundred cubic centimeters). In our cases the symptoms disappeared, as a rule, within twenty-four hours after urea was discontinued. In Case 6, however, they persisted to a lessened extent for four days thereafter. This case had a coexistent nephritis. After urea had been discon-

6. Hewlett, A. W.; Gilbert, Q. O., and Wickett, A. D.: The Toxic Effects of Urea on Normal Individuals, Arch. Int. Med. 18:636 (Nov.) 1916.

tinued on account of symptoms of intolerance, it was always possible after a short interval to resume treatment, with a smaller dose, without the production of untoward symptoms.

Most of the patients, although not all, complained of thirst while under urea medication; however, this was never severe enough to interfere with the continuation of treatment. Headache was an occasional but never a severe cause of complaint. It is not certain that any of these symptoms were entirely due to urea administration; the benefits derived from the urea were marked and in our judgment far outweighed the discomfort entailed.

Changes in Urinary Constituents.

Urea.—A study of the rate of urea excretion shows that this ran parallel with the water excretion. The amount gradually increased for two or three days after treatment was begun and thereafter remained practically constant as long as the dosage remained constant, irrespective of the time it was continued. With larger doses the amount of urea excreted was increased. Cessation of treatment rapidly reduced the output of urea to its previous level. The increased excretion of urea began within from one and one-half to three hours after its administration (Table 2). Later in the day, after a second dose of urea had been given, the amount excreted was still greater (Table 3). On the following morning much less urea was excreted, the amount depending largely on the dosage. With the smaller doses, the quantity of urea in the urine seventeen hours after the last dose was only raised to a slight extent over the amount excreted before urea was given. With the larger doses, however, an increased output was still maintained at this time (Table 1).

Chlorid.—As a rule more chlorid is excreted during urea administration than during the control period. The changes in amount are slight, however, and show very little regularity.

Abnormal Urinary Changes.—We have seen no evidence of kidney irritation as shown by the presence of albumin, red blood cells or casts. When these were present before treatment was begun they became progressively reduced in amount and in almost every case disappeared.

TABLE 1.

Effect of Urea on Urine Volume and Body Weight; the Blood Urea Nitrogen from Fifteen to Seventeen Hours after Urea Was Given, with the Rate of Urea and Urine Excretion at This Time.

Case	Disease	Age	Dose of Urea (Gm.)	Number of Days on Which Urea Was Given	Number of Days Without Urea	Weight at End of Period (Kg.)	Average Daily Urine Volume (C.c.)	Chlorid Excretion in 24 Hours as Sodium Chlorid (Gm.)	Blood Urea Nitrogen per 100 C.c. (Mg.)	Rate of Urine Excretion per 24 Hours Calculated from 72 Minute Period (C.c.)	Urea Nitrogen Excretion Rate per 24 Hours Calculated from 72 Minute Period (Gm.)	Urea Excretion Index, $\frac{B\sqrt{V}W}{10D}$	Remarks
1	Aortic disease (syphilitic); auricular fibrillation	47	..	..	..	90.6	518	1.63	13.1	500	4.64	54.2	Before urea
			30	7	..	87.6	1,069	2.47	....	.....	....	....	Novasurol, 1 cc. on second day*
			..	..	4	86.4	848	1.08	14.7	560	5.70	56.0	
			30	8	..	84.3	1,280	1.39	....	.....	....	....	
			45	5	..	86.0	1,544	0.77	25.8	1,460	18.20	63.2	
			45	10	..	85.2	1,370	0.86	35.8	1,680	24.32	56.7	
			..	..	1	85.5	875	1.65	37.6	.....	....	....	
			60	14	..	85.5	1,558	0.62	....	1,520	23.70	55.3	
2	Mitral stenosis; auricular fibrillation	31	..	..	..	53.4	585	2.70	10.5	340	1.64	36.6	Before urea
			30	6	..	53.8	980	3.09	15.8	720	9.34	95.2	
			30	4	..	54.0	931	2.15	....	.....	....	....	
			..	..	3	54.4	743	3.96	10.6	888	5.62	76.9	
			30	11	..	53.9	985	3.15	18.8	696	9.68	84.3	
3	Mitral stenosis; auricular fibrillation	37	..	..	..	50.8	385	0.08	11.4	656	1.97	29.9	Before urea
			30	27	..	51.4	1,012	0.27	22.7	648	11.10	85.1	
			30	4	..	52.5	914	0.21	25.5	760	10.60	66.8	

No.	Diagnosis	Treatment											
4	Mitral stenosis; auricular fibrillation	Before urea	20	:	:	:	41.1	447	1.70	14.3	460	4.10	69.2
		Novasurol, 2 cc. on fifth day*		30	6	:	38.2	863	2.37	24.7	780	4.64	34.8
				30	12	:	38.3	831	2.52	28.1	544	9.05	71.5
				:	:	4	38.0	573	0.90	10.6	780	9.64	84.0
				:	:	4	38.3	508	2.24	8.2	700	4.57	109.1
				30	11	:	37.2	845	2.48	....	....	....	....
5	Exophthalmic goiter; auricular fibrillation; ascites; slight edema of the legs	Novasurol, 2 cc. on fourth day*	46	:	:	:	44.9	688	2.86	15.5	1,000	4.72	47.0
				30	4	:	44.0	1,423	3.65	19.4	900	8.44	70.9
		Before urea		30	9	:	41.8	1,199	2.69	....	....	....	....
				:	:	6	43.8	640	1.28	....	....	....	35.6
				30	5	:	43.1	1,413	3.42	24.3	860	5.19	....
				30	5	:	42.5	1,298	3.47	....	....	....	....
				:	:	2	42.4	964	2.12	6.6	560	2.31	31.5
				:	:	5	44.1	940	1.81	....	....	....	....
				30	22	:	42.7	1,339	3.89	....	....	....	....
				45	15	:	42.2	1,556	1.70	36.6	560	6.62	37.3
				:	:	5	42.0	1,125	0.51	....	....	....	....

* Days on which novasurol was effective are not included.

Changes in the Blood.

Urea.—The blood urea nitrogen was increased by taking urea. The extent of the increase varied with the dose (chart). When a certain amount of urea was given on succeeding days in divided doses, the hours of administration being the same on these days, the concentration of urea in the blood *at a given time* rose for a few days, until a certain height was reached. It continued at about this level however long this dosage was continued. As soon as the administration of urea was discontinued, the amount of urea nitrogen in the blood rapidly fell to its previous level. The volume of urine and urea excretion reflected the concentration of the urea in the blood. Seventeen hours after the last dose the urea in the blood was still raised—the height varying with the amount administered—but the increase was not great (Table 1). The urea given must have been rapidly absorbed for the amount of urea nitrogen in the blood increased within three hours (Table 2).

Plasma Chlorid.—The chlorid in the plasma remained practically unaltered.

INDEX OF UREA EXCRETION.

A study of kidney function by the urea excretion index of Austin, Stillman and Van Slyke[7] is of considerable interest. These authors have made a study of the changes in the rate of urea excretion with varying concentrations of urea in the blood and with varying urine volumes. They have expressed the relationships between these factors in the following equation: $K = \dfrac{10\,D}{B\sqrt{VW}}$, in which D represents urea output (as grams per twenty-four hours), B the blood urea (as grams per liter), V the volume output (as liters per twenty-four hours), W the body weight (as kilograms) and K the excretory constant. For the normal subject K varies between 45 and 105. In kidney disease, values varying between 3 and the normal values have been observed.

With the exception of Case 1, all cases in the series fell below the minimum normal value of 45 on some occasion. In Case 6, this sub-

7. Austin, J. H.; Stillman, E., and Van Slyke, D. D.: Factors Governing the Excretion Rate of Urea, J. Biol. Chem. 46:607 (March) 1921.

TABLE 2.

Effect of Urea on Urine Volume and Body Weight; the Blood Urea Nitrogen Shortly after the Morning Dose (One and a Half to Three Hours) during Time Urea Was Given Twice Daily, with the Rate of Urea and Urine Excretion at This Time.

Case	Disease	Age	Dose of Urea	Number of Days on Which Urea Was Given	Number of Days Without Urea	Weight at End of Period	Average Daily Urine Volume	Chlorid Excretion in 24 Hours as Sodium Chlorid	Blood Urea Nitrogen per 100 C.c.	Rate of Urine Excretion per 24 Hours Calculated from 72 Minute Period	Urea Nitrogen Excretion Rate per 24 Hours Calculated from 72 Minute Period	Urea Excretion Index, $\frac{B\sqrt{W}}{10\,D}$	Remarks
			Gm.			Kg.	C.c.	Gm.	Mg.	C.c.	Gm.		
2	Mitral stenosis; auricular fibrillation	31	..	..	..	53.4	585	2.70	10.5	340	1.64	36.6	Before urea
			30	32	..	54.9	837	2.73	24.4	1,336	23.50	114.0	Received 15 gm. of urea 1½ hours before bleeding
			..	..	5	54.6	461	1.15	10.4	576	5.24	90.8	
			30	10	..	55.4	1,017	2.21	25.5	1,392	23.45	106.6	
3	Mitral stenosis; auricular fibrillation	37	..	..	..	50.8	385	0.08	11.4	656	1.97	29.9	Before urea
			30	2	..	51.0	940	0.50	25.1	1,056	16.47	89.5	Received 15 gm of urea 3 hours before bleeding
			30	23	..	52.3	1,043	0.17	36.8	864	16.94	69.4	

TABLE 3.

Effect of Urea on Urine Volume and Body Weight; the Blood Urea Nitrogen One and a Half Hours after the Second Dose during Time Urea Was Being Given Thrice Daily, with the Rate of Urea and Urine Excretion at This Time.

Case	Disease	Age	Dose of Urea Gm.	Number of Days on Which Urea Was Given	Number of Days Without Urea	Weight at End of Period Kg.	Average Daily Urine Volume C.c.	Chlorid Excretion in 24 Hours as Sodium Chlorid Gm.	Blood Urea Nitrogen per 100 C.c. Mg.	Rate of Urine Excretion per 24 Hours Calculated from 72 Minute Period C.c.	Urea Nitrogen Excretion Rate per 24 Hours Calculated from 72 Minute Period Gm.	Urea Excretion Index, $\frac{10\ B\sqrt{VW}}{D}$	Remarks
6	Mitral stenosis; aortic disease (rheumatic); chronic nephritis; ascites; edema of the legs	47	..	..	..	69.8	776	2.20	24.1	1,080	3.14	16.4	Blood pressure 180 systolic, 90 diastolic
			30	4	..	67.1	1,129	2.30	43.2	1,700	10.67	24.7	
			30	4	..	64.7	1,042	2.39	43.2	1,480	....	....	
			45	3	..	62.4	1,458	2.47	60.9	1,900	10.30	16.0	
			45	4	..	61.0	1,416	1.77	86.6	1,120	9.16	13.0	
			45	3	..	59.7	1,269	....	89.0	1,720	14.15	15.8	
			..	..	4	58.9	555	....	34.3	700	6.09	27.7	
			..	..	2	58.6	226	....	34.9	560	8.89	44.4	
7	Chronic myocarditis; auricular fibrillation; edema of the legs	60	..	..	..	57.6	1,490	12.84	16.6	840	6.57	62.8	
			30	5	..	49.6	2,338	9.89	39.8	2,500	....	....	
			45	3	..	48.1	1,569	3.50	53.8	2,100	14.68	37.0	
			45	4	..	47.2	1,285	1.00	60.0	1,900	10.05	17.7	
			..	..	2	47.5	726	....	15.8	960	6.25	58.7	
			..	..	5	48.7	980	1.06	21.0	1,500	3.82	21.6	

8	Mitral stenosis; ascites; edema of the legs	47										Nine days after previous determination
			:	:	30	:	55.6	344	0.71	21.4	680	6.14	48.6
			:	:	30	:	53.6	615	1.00	16.9	400	6.69	87.4
			2	30	:	53.5	575	0.33	54.0	880	....	....
			3	30	:	53.4	692	0.46	41.1	1,060	17.99	59.4
			4	45	:	53.1	900	0.52	56.9	1,600	14.41	28.6
			3	45	:	52.9	961	0.89	57.4	3,160	18.90	25.9
			4	60	:	51.9	1,050	....	78.2	2,500	19.99	22.6
			3	60	:	51.1	1,115	....	74.9	2,520	19.94	23.4

normal value was chiefly due to a chronic nephritis. In Cases 2, 3 and 6 there was a marked tendency of the index to rise as treatment was continued. This rise was coincident with a marked improvement in the clinical condition. Two cases in the series (Cases 7 and 8) showed low indexes during treatment. Case 8 showed a primary rise of the index with a subsequent fall to a still lower level. It will be recalled that this case did not improve with treatment. Case 7, however, showed a marked improvement, and finally the patient went home with compensation reestablished. The two markedly subnormal indexes found in his case are unexplained. There was no evidence of nephritis.

The general tendency of the index to rise during urea feeding does not occur in normal subjects.[7] It would seem to reflect a general improvement in the clinical condition of the cases under urea treatment. It gives further evidence against the possibility of kidney damage due to urea.

Comment.

Many of the most troublesome symptoms of cardiac decompensation are due to the accumulation of fluid in the tissues, in body cavities and in parenchymatous organs. The most important causal factor is certainly the failure of the efficiency of the myocardium, and it is to the amelioration of this that our first efforts must be directed. But frequently in the more advanced cases of heart disease, the maximum efficiency that can be obtained by treatment of the heart per se does not suffice to remove edema nor to maintain the patient free from symptoms. In the cases treated with urea a marked improvement in the clinical condition of the patient took place which could be assigned to the action of urea. The treatment succeeded in maintaining an adequate urine output and also in removing edema. As soon as the administration of urea was stopped, the urinary output immediately fell and the clinical condition became worse. When treatment was resumed an improvement again took place. We have studied some of our cases for months and have found that a particular dose will give the same daily urinary output with only slight variations throughout the period investigated. In these cases it has seemed that the maintenance of an adequate water excretion has been instrumental

not only in preventing symptoms but also in avoiding a relapse. We have also prescribed its use at home by patients who have been discharged from the hospital after treatment for decompensation. Here again careful treatment of the cardiac condition is of prime importance, but it seems that urea has been of great assistance in keeping these patients comfortable through the maintenance of a normal water balance.

Urea is rapidly absorbed and an increase immediately occurs in the height of the urea in the blood. The mean level of the urea in the blood is dependent on the dosage, and the relationship between them is fairly constant in any particular case. The amount of urea excreted depends on the blood urea, so that with constant urea administration a state of equilibrium is reached between the intake and the output. During urea diuresis the excretion of water runs more or less parallel with that of urea, so that urine volume reflects the concentration of urea in the blood. Undoubtedly the explanation of the diuresis is that the excess of urea circulating in the blood is excreted by the kidney, and during the process carries with it a considerable amount of water.

CONCLUSIONS.

1. Urea was given in doses of from 30 to 60 gm. a day to eight patients with advanced heart failure and was followed by a marked increase in urine volume. The drug was particularly useful in cases in which an adequate water excretion was not maintained after the edema fluid had been removed by other measures. In some cases it relieved the edema when other remedies had failed.

2. The increase in urine output varied with the dose and followed closely the curve of urea excretion. With continuous administration the daily urine volume was maintained at an almost constant level. The response after administration was rapid, but the effect passed off in a short time unless the dose was repeated.

3. The changes in urine volume and in urea excretion were dependent on the concentration of the urea in the blood.

4. In several of the cases there was a subnormal index of urea excretion which seemed ascribable to advanced heart failure. The index tended to improve with urea administration, along with a general improvement in the clinical condition.

5. Toxic symptoms of any significance did not take place.

6. From these observations we would suggest that urea is a useful diuretic in cases of heart failure with edema in which treatment of the cardiac condition has failed to remove the edema or maintain an adequate water excretion.

THE SOLUBLE SPECIFIC SUBSTANCE OF A STRAIN OF FRIEDLÄNDER'S BACILLUS.

PAPER I.

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In 1921 Toenniessen (1), working on the capsular material of a strain of Friedländer's bacillus, reported the isolation of a snow-white, non-reducing, substantially nitrogen-free polysaccharide which gave a red color with iodine. After hydrolysis the resulting reducing sugars were found to yield an osazone which Toenniessen believed to be that of galactose. These findings were subsequently confirmed by Kramár (2). More recently (3) the so called "soluble specific substance" of the Pneumococcus, first observed by Dochez and Avery (4), has been identified with the polysaccharide portion of the organism. It seemed not unlikely, therefore, that Toenniessen's carbohydrate from the Friedländer bacillus would also possess specific properties, and an investigation of this point was undertaken. Meanwhile the correctness of this surmise has been indicated in a preliminary report by Mueller, Smith, and Litarczek (5), who isolated carbohydrate-containing material with a nitrogen content of 1.3 per cent from a strain of Friedländer's bacillus, and showed that this substance at high dilutions caused precipitation of homologous immune serum.

In the present work a strain of Friedländer's bacillus recovered from a spontaneous guinea pig infection was used. This will be referred to as the E strain, its laboratory designation. From this strain, by a procedure essentially the same as that used in the case of the Pneumococcus (3, 6), a nitrogen-free polysaccharide with specific properties of a most unusual nature has been obtained.

EXPERIMENTAL.

1. Isolation of the Soluble Specific Substance of Friedländer's Bacillus.

Autoclaved washings from 60 to 70 Blake bottles of 48 hour cultures of the E strain of Friedländer's bacillus on solid agar at pH 7.6 were diluted to 3 liters and run through a Sharples centrifuge to remove bacterial debris. The resulting liquid, made slightly alkaline, was treated with 60 gm. of sodium acetate and 4.5 liters of alcohol. After several hours the flocculent precipitate was separated by centrifugation and was dissolved in 2 liters of water. The solution was again centrifuged and reprecipitated as before. With each alkaline precipitation protein material, but no polysaccharide,[1] was eliminated, until finally a biuret-free product was secured. Usually from four to six such precipitations were necessary. At this point the solution of the substance, at a volume of 2 liters, was filtered through a Berkefeld V candle, evaporated to 500 cc., and precipitated with an excess of hot barium hydroxide solution saturated at 60–70°.

The barium salt was suspended in 500 cc. of water and treated with a slight excess of sulfuric acid. The barium sulfate was centrifuged off, washed with very dilute sulfuric acid, and the clear supernatants were neutralized and concentrated to 100 cc. *in vacuo.* The polysaccharide was then precipitated at 0° by the addition of 35 cc. of 1:1 hydrochloric acid and 225 cc. of redistilled alcohol. After standing for 1 hour the precipitate was centrifuged off in the cold, redissolved in 100 cc. of water, and reprecipitated under the same conditions. The final product was washed free from chloride ion with acetone on a hardened filter paper and dried over sulfuric acid *in vacuo.* The yield was 2 to 2.5 gm.

A number of preparations were isolated in this way. The properties of the polysaccharide thus obtained are shown in Table I. The methods of purification employed were the same in all preparations except in the case of Nos. 103 I and 103 II, in which, instead of using agar media, 2 day cultures grown in glucose-aminoid-peptone broth at pH 7.6 were employed. In these cases the initial steps were identical with those employed in the preparation of the soluble specific substances of Types II and III pneumococcus. One volume of alcohol was found to be sufficient for the initial precipitation of the soluble substance. The usual three layer separation was obtained by centrifugation and only the middle layer contained active material. The remainder of the process of purification was as outlined above.

[1] In this preparation complete precipitation of the polysaccharide was followed by tests of portions of the hydrolyzed mother liquors with Fehling solution.

The properties of the various preparations were remarkably uniform, as shown by Table I.

The soluble specific substance of the E strain of Friedländer's bacillus is a white fluffy amorphous powder with acid properties strong enough to turn wet Congo red paper blue when a few particles are dusted upon it.   After the substance is dry it dissolves with difficulty in water, but passes readily into solution on neutralization with dilute sodium hydroxide.   A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, or phosphotungstic acid,

TABLE I.

Soluble Specific Substance of the Friedländer Bacillus (E.)

Preparation No.	$[\alpha]_D$	Acid equivalent.	Ash.	C	H	N	Percentage of reducing sugars on hydrolysis.	Highest dilution giving a precipitate with immune serum.	
								Anti-Friedländer serum E.	Antipneumococcus serum Type II.
101	+100.0°	670	0.0	44.6*	6.1	0.0	73.0	1:2,000,000	1:2,000,000
103 I	+102.5°	674	0.0			0.0	72.4	1:2,000,000	1:2,000,000
103 II	+100.0°	704	0.0			0.0	70.0	1:2,000,000	1:2,000,000
104	+100.0°	722	0.0			0.06	73.0	1:2,000,000	1:2,000,000
104 A	+100.0°	685	0.0			0.0	72.0		
105 A	+100.0°	706	0.0			0.66	72.0	1:2,000,000	1:2,000,000
105 B†	+101.5°	674	0.0			0.2	73.0		
105 Ad	+100.0°	716	0.0			0.0	78.0		
25 A‡	+ 70.2°	1302	0.35	45.8	6.4	0.0	68.4		1:6,000,000

* Theory for $(C_6H_{10}O_5)_x$, C = 44.4, H = 6.2.

† This represented a residue which failed to pass through a Berkefeld filter.

‡ A preparation of Type II pneumococcus soluble specific substance given for comparison.

but yields precipitates with barium hydroxide and with both neutral and basic lead acetate.   It gives no color with iodine-potassium iodide solution.   In several of the preparations the polysaccharide was obtained free from nitrogen.   When any traces of nitrogen remained these could be removed by the additional methods of purification outlined below.   Micro Kjeldahl determinations (Pregl) were made on samples as large as 30 to 40 mg.

In many of its precipitation reactions and in yielding glucose on hydrolysis (see below) the substance resembles the soluble specific

substance of Type II pneumococcus, although in its present state of purity there are points of difference between the two products (compare with 25 A, Table I; also reactions with barium hydroxide and neutral lead acetate). This similarity in chemical properties led to a test of the specific reaction of the substance with Type II antipneumococcus serum. Under these conditions a "specific" immune precipitate resulted, while no reaction occurred in the presence of antipneumococcus sera Types I and III. This unusual phenomenon and its immunological significance are treated in detail in the following paper.

2. Attempts at Further Purification of the Specific Substance.

Since the specific substance yielded a precipitate with uranyl nitrate, a property common to the analogous substances of the three types of Pneumococcus, a portion was precipitated with this salt to see whether additional purification could be effected.

To a neutralized solution of 0.4 gm. of Preparation 104 in 80 cc. of water 20 cc. of 5 per cent uranyl nitrate solution were added. The heavy precipitate which formed was separated after 24 hours by centrifugation. The supernatant fluid gave a negative specificity test and only a faint reaction for uranyl ions. The precipitate was suspended in water and dissolved by addition of the least possible amount of normal hydrochloric acid. Molar potassium dihydrogen phosphate solution was then added until no further precipitate occurred. The uranyl phosphate was centrifuged off and the supernatant liquid, containing the specific substance, was concentrated *in vacuo* to 50 cc. and precipitated by the addition of 1 gm. of sodium acetate and 100 cc. of alcohol. The precipitate was dissolved in 20 cc. of water and the solution cooled to 0° and treated with 3 cc. of hydrochloric acid and 60 cc. of redistilled alcohol. After 1 hour the substance was separated from the slightly active supernatant liquid by centrifugation, redissolved in 20 cc. of water, and thrown into 200 cc. of acetone. The yield was 0.27 gm.

A comparison of this preparation (104 A, Table I) with the original material (104) shows that the only significant change was the removal of the last traces of nitrogen.

The highly adsorbent Alumina A prepared according to Willstätter and Kraut (7) was also used for further purification.

A solution of 0.5 gm. of Preparation 105 A was shaken with 1.5 gm. (calculated as Al_2O_3) of Type A alumina in a volume of 250 cc. at pH 5.0. At the end of 1

hour the alumina, containing the adsorbed polysaccharide, was centrifuged off from the now specifically inactive liquid. The precipitate was extracted once with 250 cc. of N/100 sodium hydroxide for 2 hours and a second time with 200 cc. of N/5 sodium carbonate solution. After the extracts were neutralized with acetic acid they were concentrated *in vacuo* to a volume of 50 cc. and the polysaccharide was precipitated with 100 cc. of alcohol. The specific substance was centrifuged off, dissolved in 25 cc. of water, and the solution was centrifuged from a small amount of insoluble alumina and finally treated at 0° with 5 cc. of 1:1 hydrochloric acid and 60 cc. of redistilled alcohol. After 2 hours the precipitate was centrifuged off, dissolved in 15 cc. of water, and poured into 10 volumes of cold acetone. The product was finally filtered and washed with acetone until free from chloride ion. 0.32 gm. of dry material was recovered.

A comparison of the product (105 Ad, Table I) with the original material (105 A) shows that adsorption and recovery of the polysaccharide had little effect other than elimination of the nitrogen present.

3. Isolation of Glucosazone and Potassium Hydrogen Saccharate from the Hydrolysis Products of the Specific Substance.

1 gm. of Preparation 101 was dissolved in 60 cc. of normal sulfuric acid and the solution was boiled for 5 hours, until no further increase in reducing sugars could be observed. After dilution to 250 cc. the sulfuric acid was quantitatively removed by barium hydroxide solution. The barium sulfate was centrifuged off and the supernatant fluid was concentrated to 30 cc., boiled with Norite, filtered, and finally treated with 1.4 gm. of phenylhydrazine acetate. After heating on the water bath for 1 hour the crystalline osazone which had formed was filtered off. A second fraction was obtained on heating the solution further. Since both portions appeared to be quite pure they were combined, washed with a small amount of methyl alcohol to remove tar, filtered off, and dried. 0.25 gm. of substance was recovered, melting at 201° with decomposition. After recrystallization from 60 per cent ethyl alcohol the decomposition point was 203–204°. A mixture of this compound with the osazone prepared from pure glucose also melted at 203–204°.

0.1000 gm. of substance gave 13.40 cc. of N_2 at 23°C., 761.7 mm.

Calculated for $C_{18}H_{22}O_4N_4$; N, 14.45 per cent. Found: N, 14.49 per cent.

0.0576 gm. of the compound dissolved in 5 cc. of pyridine-alcohol mixture had an initial $[\alpha]_D$ of $-67.9°$ which decreased after standing 4 days to $-24.2°$.

From the analysis, melting point, and direction of mutarotation (8) the substance is undoubtedly glucosazone. The identification of glucosazone limits the sugar formed on hydrolysis of the specific substance either to glucose, fructose, or mannose.

In order to ascertain which of these sugars was actually present 1 gm. of Preparation 103 II was boiled for 5½ hours under a reflux condenser with 100 cc. of one-half normal nitric acid. The hydrolysis products had a specific rotation of +75°. The solution was concentrated *in vacuo* to 5 cc. and to it was added 1 cc. of concentrated nitric acid. After 24 hours at room temperature the mixture was boiled for 2.5 minutes and then quickly evaporated, with stirring, on a large watch-glass over a boiling water bath. A thick paste was obtained which was twice evaporated with a small quantity of water, to expel the last traces of nitric acid, and was finally dissolved in 5 cc. of water. The solution was made strongly alkaline with 40 per cent potassium hydroxide solution and then acidified with glacial acetic acid. After 5 hours the crystals of potassium acid saccharate which had separated were filtered off and recrystallized from 2 cc. of boiling water.

0.0494 gm., ignited with sulfuric acid, yielded 0.0173 gm. K_2SO_4.

Calculated for $COOH(CHOH)_4COOK$; K, 15.75 per cent. Found: 15.70 per cent.

It is thus evident that as in the case of the specific substance of the Type II pneumococcus glucose is the principal sugar from which the specific substance of Friedländer's bacillus (Strain E) is built up.

DISCUSSION.

It is clear from the foregoing that the E strain of Friedländer's bacillus yields, on fractionation, a nitrogen-free polysaccharide with specific properties of the order possessed by the soluble specific substances of the three fixed types of Pneumococcus. It is a strong acid with an equivalent value of about 685, sparingly soluble in water after drying, but yielding soluble alkali salts. The specific optical rotation is +100°. The polysaccharide itself is non-reducing, but on hydrolysis with mineral acid yields reducing sugars, among which glucose has been shown to be present. In the present instance, as in those of the pneumococci, carbohydrate and specific function are apparently inseparable, and the isolation of a fourth specific substance of this nature adds additional weight to the growing mass of evidence that the soluble specific substances of microorganisms are often actually polysaccharides (*cf.*(5) also Mueller and Tomcsik (9)[2]).

[2] These investigators isolated specific polysaccharide material from Friedländer's bacillus and yeast. Since the present communication was accepted for publication Laidlaw and Dudley (*Brit. J. Exp. Path.*, 1925, vi, 197) have described a specifically precipitating, non-nitrogenous carbohydrate isolated from tubercle bacilli.

While the specific substance of the E strain, as purified up to the present, has properties which set it apart from the three analogous substances of the fixed types of Pneumococcus there is nevertheless a certain resemblance to that of the Type II pneumococcus, a resemblance extending even to precipitation with Type II antipneumococcus serum. The immunochemical relationships of these otherwise widely different microorganisms are treated in the succeeding paper.

Work on the specific substance of the E strain is being continued and the soluble specific substances of other strains of the Friedländer bacillus are also under investigation.

SUMMARY.

1. A method is given for the isolation of a specifically reacting nitrogen-free polysaccharide from the so called E strain of Friedländer's bacillus.

2. The properties of this polysaccharide are described.

BIBLIOGRAPHY.

1. Toenniessen, E., *Centr. Bakt.*, *1. Abt.*, *Orig.*, 1921, lxxxv, 225.
2. Kramár, E., *Centr. Bakt.*, *1. Abt.*, *Orig.*, 1922, lxxxvii, 401.
3. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
4. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
5. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.
6. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 727.
7. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, 1923, lvi, 149.
8. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xx, 429.
9. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343.

THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S BACILLUS.

Paper II. Chemical and Immunological Relationships of Pneumococcus Type II and of a Strain of Friedländer's Bacillus.

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The preceding paper (1) records the initial attempts to isolate and purify the soluble specific substance of the so called E strain of a bacillus of the Friedländer group. The organism was originally isolated from a spontaneous epidemic of pneumonia among stock guinea pigs. The specifically reactive substance which has been isolated appears to be a nitrogen-free polysaccharide differing in many respects from the soluble specific substances recovered from the three fixed types of Pneumococcus (2). However, as in the case of the soluble specific substance of Pneumococcus Type II, only glucose could readily be identified among the products of hydrolysis. Moreover, the percentage of reducing sugars on hydrolysis is almost the same in both substances, and it therefore seemed possible that both of them might in reality be identical and that the differences observed might be due to impurities. In the preceding paper procedures were outlined which were designed to eliminate any impurities present in the Friedländer polysaccharide, but the employment of these methods only resulted in recovery of the original substance practically unaltered.

A comparison of the specific substance obtained from Type II pneumococcus with that isolated from the E strain of Friedländer's bacillus shows that both polysaccharides rotate the plane of polarized light to the right. In the case of the Friedländer substance the specific rotation is $+100°$, while the Type II pneumococcus sub-

stance rotates the plane of polarized light about +74°. Both sub-
stances have acidic properties, but the Friedländer specific substance
has an acid equivalent approximately one-half that of the Type II
polysaccharide. Neither of the two products gives glucuronic
acid tests as do the specific substances of Types I and III pneumo-
coccus. Both polysaccharides fail to form precipitates when treated
with solutions of silver nitrate, copper sulfate, or phosphotungstic
acid; both are precipitated by solutions of uranium nitrate and
basic lead acetate. Whereas the Type II pneumococcus specific
substance gives no precipitate with either barium hydroxide or
neutral lead acetate, the Friedländer polysaccharide is precipitated
by both of these reagents.

Because the two specific substances, although of widely different
biological origin, resemble each other so closely in some of their
chemical properties, the Friedländer polysaccharide was tested
with Type II antipneumococcus serum, and a precipitin reaction was
found to occur. On the other hand, there was absence of precipi-
tation when this substance was tested with antipneumococcus
serum of the other two fixed types. It then became necessary to
determine as far as possible the immunological relationships not
nly of the soluble substances of the E strain of the Friedländer
cillus and of Type II pneumococcus, but of the microorganisms
emselves. The present paper deals with the facts so far ascer-
tained in this study.

EXPERIMENTAL.

Microorganisms.—The strain of Gram-negative bacillus from which the
specific polysaccharide was derived corresponds in its cultural and biological
reactions to organisms of the *encapsulatus* group, and for laboratory purposes is
referred to as the E strain. Five other strains, four of which, as will be shown
later, are similar in their serological reactions to the original organism, have been
used in this study. It does not seem essential at this time to enter into a detailed
description of the individual cultural characteristics of each strain;—they all
possess in common the property of luxuriant growth on ordinary media with the
elaboration of the abundant gummy, mucoid material characteristic of the group.

Methods.—Rabbits were immunized by the intravenous injection of suspensions
of heat-killed organisms; *small* doses were administered every day for 6 days, and
they were repeated after an interval of a week. Three courses of injections were
given, and the animals were bled 9 days following the last inoculation.

The agglutination and precipitin tests were done as previously described for Pneumococcus. The protective power of type sera against Friedländer's bacilli and pneumococci was tested by injecting white mice intraperitoneally with varying doses of the virulent organisms and at the same time injecting a fixed quantity (0.2 cc.) of immune serum. All animals were observed for at least 10 days following injection, and those surviving this period were considered effectively protected.

Agglutination.—Immune rabbit sera were prepared against two strains of Friedländer's bacillus. One of these was the culture E from which the specific polysaccharide was isolated. The second culture (Sc) was isolated from the blood of a patient suffering from pneumonia. The study of the agglutinin reactions of these two immune sera afforded an opportunity, first, to establish the specific relationship of these two strains to each other and to other organisms of the group; and second, to confirm the immunological relationships between Type II pneumococcus and the E strain.

It immediately becomes evident from Table I that, as others have previously observed, the *encapsulatus* group does not consist of organisms which are immunologically identical. The two strains (E and Sc) chosen at random are antigenically distinct one from the other. Observations by Julianelle (3) indicate, however, that these two strains are representatives of two distinct but not unusual immunological types, since of forty strains of Friedländer's bacillus which he has tested, seventeen were found to be immunologically identical with either Strain E or Strain Sc. It is not the purpose of this paper, however, to deal with the serological relationships within the Friedländer group. Of more immediate interest is the fact, as shown by the experiments recorded in Table I, that the Friedländer Type E strain reacts in antipneumococcus Serum II and that Type II pneumococcus is agglutinated in serum produced by immunization with the E strain of Friedländer's bacillus. An immunological similarity, therefore, exists between these two biologically remote organisms, and this relationship is reciprocal. The experiment also shows that there is no immunological relationship between Pneumococcus Type II and the other strain of Friedländer's bacillus tested (Sc), which, although a typical organism of the *encapsulatus* group, is immunologically distinct from Strain E.

TABLE I.

Agglutination Reactions of Two Strains of Friedländer's Bacillus and Pneumococcus Type II in Homologous and Heterologous Sera.

Culture.	Immune sera.														
	Anti-Friedländer Sc.*					Anti-Friedländer E.†					Antipneumococcus Type II.‡				
	1:5	1:10	1:20	1:40	1:80	1:5	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:160
Friedländer's bacillus (Sc)......	++++	++++	+	−	−	−	−	−	−	−	−	−	−	−	−
Friedländer's bacillus (E)......	−	−	−	−	−	++++	++++	++++	++	−	+++	+++	+	−	−
Pneumococcus Type II........	−	−	−	−	−	++++	++++	++	++	−	++++	++++	+++	++	±

++++ indicates complete, compact, disk-like agglutination; +++, marked, compact, disk-like agglutination; ++, clumping—more easily broken up; +, partial clumping; ±, slight clumping; −, negative.

* Rabbit 77 D.
† Rabbit 84 D.
‡ Horse 91 A.

That other strains of Friedländer's bacilli, which are immunologically identical with Strain E, also react with antipneumococcus serum Type II just as does the E strain is shown in Tables II, III, and IV, in which are presented the results of agglutination reactions of six

TABLE II.

Agglutination of Friedländer's Bacillus and Pneumococcus Type II in Anti-Friedländer Sera.

Friedländ-er's bacillus.	Anti-Friedländer Serum E.					Anti-Friedländer Serum Sc.				
	1:5	1:10	1:20	1:40	1:80	1:5	1:10	1:20	1:40	1:80
E	++++	++++	++++	+++	−	−	−	−	−	−
K	++++	++++	+++	+++	−	−	−	−	−	−
H$_1$	++++	++++	++++	++	−	−	−	−	−	−
H$_2$	++++	++++	++++	+++	−	−	−	−	−	−
F$_6$	++++	++++	++++	++++	++	−	−	−	−	−
Sc	−	−	−	−	−	++++	++++	+	−	−
Pneumo-coccus Type II.	+++	+++	++	±	−	−	−	−	−	−

TABLE III.

Agglutination of Friedländer's Bacillus in Antipneumococcus Serum Type II.

Friedländer's bacillus.	Antipneumococcus serum.						Normal horse serum.		Salt solution.
	Type I.		Type II.		Type III.				
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	
E	−	−	++++	++++	−	−	−	−	−
K	−	−	++++	++++	−	−	−	−	−
H$_1$	−	−	++++	++++	−	−	−	−	−
H$_2$	−	−	++++	++++	−	−	−	−	−
F$_6$	−	−	++++	++++	−	−	−	−	−
Sc	−	−	−	−	−	−	−	−	−
G$_9$	−	−	−	−	−	−	−	−	−

additional strains. When the strains are immunologically related to Strain E they react with antipneumococcus serum Type II; when they are immunologically distinct (Sc and G$_9$, Table III) they fail to react. Moreover, Table III shows that the agglutination of Friedländer's bacilli of the Strain E group does not occur with anti-

pneumococcus sera of Types I and III, but only in that of Type II. This fact suggests that the immunological relationship between certain strains of the Friedländer bacillus and certain strains of Pneumococcus is dependent upon chemical similarities in the soluble specific substances of the organisms concerned. Table IV shows that while Friedländer strains of the E type all react about equally in Type II antipneumococcus serum, they do not react in as high dilution as does Pneumococcus Type II itself. This difference in the capacity of the two kinds of bacteria to react to the same degree in the same immune serum shows a lack of complete immunological identity between them, a fact presumably related to the minor chemical

TABLE IV.

Agglutination of Friedländer's Bacillus by Antipneumococcus Serum II.

Friedländer's bacillus (strains).	Antipneumococcus serum Type II.							Control.
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	
E	++++	++++	+++	+	−	−	−	−
K	++++	++++	++++	±	−	−	−	−
H₁	++++	++++	+++	±	−	−	−	−
H₂	++++	++++	++++	+++	−	−	−	−
F₆	++++	++++	++++	±	−	−	−	−
Sc.	−	−	−	−	−	−	−	−
G₉	−	−	−	−	−	−	−	−
Pneumococcus Type II.	++++	++++	++++	++++	++++	++	−	−

differences which appear to exist between the soluble specific substances of the two organisms.

Agglutinin Absorption.—Krumwiede and his associates (4) have recently published a critical and analytical review of the subject of agglutinin absorption in which they point out the significance of the method for bacterial identification. It will be important to apply this method with great exactness to the present problem, but at present we can only record the results so far obtained.

The results of observations as given in Tables V and VI indicate the failure of reciprocal absorption. Unabsorbed anti-Friedländer serum which agglutinates both the Strain E Friedländer bacillus and Pneumococcus Type II to about the same titer, loses its agglu-

TABLE V.

Agglutinin Absorption.

Culture.	Anti-Friedländer Serum E.												Normal rabbit serum control.			
	Unabsorbed.				Absorbed with Friedländer's bacillus (E).				Absorbed with Pneumococcus Type II.							
	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40
Friedländer's bacillus (E)......	++++	++++	++++	++	−	−	−	−	++++	++++	+++	+	−	−	−	−
Pneumococcus Type II......	++++	++++	++++	+	−	−	−	−	−	−	−	−	−	−	−	−

++++ indicates complete, compact agglutination; −, no reaction.

TABLE VI.

Agglutinin Absorption.

Heat killed suspension of	Anti-Friedländer Serum E.											
	Unabsorbed.						Absorbed by Pneumococcus II.					
	1:5	1:10	1:20	1:40	1:80	1:160	1:5	1:10	1:20	1:40	1:80	1:160
Pneumococcus Type II......	++++	++++	++++	++	−	−	−	−	−	−	−	−
Friedländer's bacillus (E)......	++++	++++	+++	+	−	−	+++	++++	++	++	−	−

Heat-killed suspension of	Antipneumococcus serum Type II.															
	Unabsorbed.								Absorbed with Friedländer's bacillus (E).							
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Pneumococcus Type II......	++++	++++	++++	++++	+++	++	+	−	++++	++++	++++	++++	++	+	±	−
Friedländer's bacillus (E)......	++++	++++	+	±	−	−	−	−	−	−	−	−	−	−	−	−

tinins for both organisms when absorbed with the homologous strain of Friedländer's bacillus. On the other hand, absorption of the same serum with Pneumococcus Type II removes only the agglutinins for the absorbing organism and leaves the antibodies for the Friedländer strain only slightly reduced. Similarly, absorption of antipneumococcus serum Type II with Pneumococcus of the homologous type results in complete removal of agglutinins for both organisms, while absorption of the same serum with the E strain of Friedländer's bacillus takes out the antibodies for this organism and only slightly reduces the agglutinating power of the serum for Type II pneumococcus.

TABLE VII.

Precipitin Reactions: Anti-Friedländer Serum E against Carbohydrate Fraction Derived from Homologous Strain.

Anti-Friedländer Serum E.	Carbohydrate of Friedländer's bacillus (E).						
	1:50,000	1:100,000	1:250,000	1:500,000	1:1,000,000	1:2,000,000	1:4,000,000
0.2 cc.							
Rabbit 84.....................	++++	++++	+++	+++	++	+±	±
" 83.....................	++++	++++	+++	+++	+±	+	−
" 82.....................	++++	++++	+++	++	+±	+	−
" normal..............	−	−	−	−	−	−	−
Antipneumococcus serum Type II........................	+++	+++	+++	+++	+++	++	±

Precipitin Reactions.—Immunization of rabbits with Friedländer's bacillus (Strain E) engenders antibodies which not only agglutinate organisms of the same type, but which precipitate the soluble specific polysaccharide derived from the bacterial cell. As in the case of the specific polysaccharide of Pneumococcus the reactivity of the Friedländer substance is exhibited in dilutions as great as 1:2 million. This fact is evident in Table VII, in which immune rabbit sera in quantities of 0.2 cc. are shown to react with minute amounts of the carbohydrate derived from the homologous strain of Friedländer's bacillus. Just as antipneumococcus serum has been found to agglutinate Friedländer's bacilli of the type represented by Strain E, so also it reacts in precipitin tests with the Friedländer specific substance in high dilution.

Cross-precipitin reactions between the pneumococcus and Friedländer polysaccharides are shown in Table VIII. The soluble specific substances of both organisms are reciprocally reactive with the antibacterial serum of each in dilutions of 1:2 million. The physical properties of the immune precipitate formed by the union of the pneumococcus carbohydrate with the anti-Friedländer serum differ from those of the opaque disk characteristic of the other reactions in that the precipitate is less heavy, more transparent, and tends to form a gelatinous film.

The "specificity" of the reaction is illustrated in Table IX. Here, four protein-free carbohydrates prepared from four different organ-

TABLE VIII.

Cross-Precipitin Reactions.

Immune sera.	Carbohydrate of Friedländer's bacillus (E).					Carbohydrate of Pneumococcus Type II.				
	1:200,000	1:500,000	1:1,000,000	1:2,000,000	1:4,000,000	1:200,000	1:500,000	1:1,000,000	1:2,000,000	1:4,000,000
Anti-Friedländer E. Rabbit 84........	++++	+++	++	+	±	++++*	++	+	±	−
Antipneumococcus Type II. Horse 91 A..............	++++	+++	++	+	−	++++	+++	+	±	−

* Thin translucent film-like precipitate.

isms are tested in cross-precipitin reactions with the corresponding antibacterial serum of each strain. The three polysaccharides derived from the three fixed types of Pneumococcus show only the specific type reactions in antipneumococcus serum. The cross-precipitation between the Type II pneumococcus specific substance and the anti-Friedländer serum, and the reverse reactions are again evident.

Precipitin Absorption.—Aliquot portions of diluted anti-Friedländer serum were separately absorbed with the homologous culture and with Pneumococcus Type II. The absorbed antibacterial serum was then tested for precipitins against dilutions of the carbohydrates derived from each of the organisms. The results are given in Table X.

As in the case of agglutinin absorption, the anti-Friedländer serum (E) treated with the homologous bacilli loses its precipitins for both

TABLE IX.

Precipitin Reactions of Soluble Specific Substances of Pneumococcus Types I, II, III, and of Friedländer's Bacillus (Strain E).

Immune sera.	Carbohydrate fraction isolated from‡			
	Pneumococcus.			Friedländer's bacillus (E).
	Type I.	Type II.	Type III.	
Antipneumococcus*Type I............	++++	−	−	−
" " II............	−	++++	−	++++
" " III..........	−	−	++++	−
Anti-Friedländer† E.................	−	++	−	++++

− indicates no reaction; ++, delicate film-like scale; ++++, heavy, compact, disk-like precipitate.

* Immune horse serum.
† Immune rabbit serum.
‡ Isolated, purified soluble specific substance in dilution of 1:50,000.

TABLE X.

Precipitin Absorption.

Carbohydrate fraction of		Anti-Friedländer Serum E.			Control.
		Unab-sorbed.	Absorbed with Friedländer's bacillus (E).	Absorbed with Pneumo-coccus II.	Normal rabbit serum.
Friedländer's bacillus (E)	1:10.000	+++	−	++	−
	1:50,000	++++	−	+++	−
	1:100,000	++++	−	+++	−
	1:200,000	++++	−	++++	−
Pneumococcus Type II...........	1:10,000	+	−	−	−
	1:50,000	++	−	−	−
	1:100,000	++±	−	−	−
	1:200,000	+++	−	−	−

the Friedländer and Pneumococcus II specific substances, whereas absorption with Type II pneumococci removes the antibodies for the pneumococcus polysaccharide, but leaves those for the Fried-

länder substance only slightly diminished. The converse of this, in which antipneumococcus serum is absorbed with each of the two cultures, yields similar results, Pneumococcus Type II removing the precipitins for both specific substances and the Friedländer bacillus depleting the serum only of antibodies for its own substance.

Protection.—The facts brought out by the test-tube reactions of agglutination and precipitation find added confirmation in the more final proof of reciprocal protection against infection in the animal body.

The six strains of Friedländer's bacilli tested for virulence in mice have each caused a fatal infection in doses as small as 1 ten-millionth cc. The protective power of immune sera against Friedländer's bacilli and pneumococci was tested by injecting white mice intraperitoneally with increasing doses of virulent organisms together with a fixed amount of type serum. Animals surviving 10 days were considered effectively protected. All animals except the virulence controls received 0.2 cc. of immune serum.

The serum of a rabbit immunized with the Friedländer bacillus (Strain E) was first tested for its power to protect mice against infection with virulent cultures of homologous and heterologous types of the Friedländer bacillus.

The protocol given in Table XI shows that 0.2 cc. of the anti-Friedländer serum Type E protected animals against 0.1 cc. of a virulent culture of the homologous strain which without immune serum caused death of the mice in doses of one-millionth cc. On the other hand, the same serum afforded no protection in mice against a virulent heterologous Strain Sc, which, by the reaction of agglutination, was shown to be of another type.

Since the E strain of Friedländer's bacillus was found to be agglutinated and its soluble specific substance to be precipitated by antipneumococcus serum Type II, it was of interest to determine whether protection against infection with a biologically different organism was possible by the use of antipneumococcus serum Type II. The results of this experiment are given in Table XII.

It is evident from Table XII that mice inoculated with 0.2 cc. of antipneumococcus serum Type II were effectively protected against at least a thousand lethal doses of the virulent E strain of Friedländer's bacillus. As in the agglutinin and precipitin tests the

"specific" nature of this protective reaction is shown by the fact that antipneumococcus serum Type I afforded no protection whatever against infection (Table XIII).

TABLE XI.

Protective Action of Anti-Friedländer Serum (E) against Friedländer's Bacillus of Homologous and Heterologous Types.

Anti-Friedländer Serum E.	Friedländer's bacillus.			
	Strain E.		Strain Sc.	
	Amount of culture.	Result.	Amount of culture.	Result.
cc.	*cc.*		*cc.*	
0.2	0.1	S.	0.1	D. 17
0.2	0.01	"	0.01	" 18
0.2	0.001	"	0.001	" 22
0.2	0.0001	"	0.0001	" 22
0	0.00001	D. 40	0.00001	" 30
0	0.000001	" 18	0.000001	" 23

In this and the following tables S. indicates survived; D., death, the numerals representing the hours before death of the animal occurred.

TABLE XII.

Protective Action of Antipneumococcus Serum Type II against Friedländer's Bacillus (E).

Friedländer's bacillus (E).	Antipneumococcus Serum II.		Virulence controls.
	Amount.	Result.	
cc.	*cc.*		
0.001	0.2	S.	
0.0001	0.2	"	
0.00001	0.2	"	
0.000001	0.2	"	
0.0000001	0.2	"	
0.00001	0		D. 51
0.000001	0		" 41
0.0000001	0		" 44

The anti-Friedländer serum prepared by immunization with Strain E protected mice against a hundred thousand lethal doses of another strain (K), which, by agglutination, was classified as belonging to the same type as E. Similarly antipneumococcus serum

Type II afforded protection against the K strain just as it did against the E strain; a fact which emphasizes again the immunological similarity of Friedländer's bacillus of this type to Type II pneumococcus. The same protocol (Table XIII) shows that antipneumococcus serum Type I is wholly without protective action against this type of Fried-

TABLE XIII.

Protective Action of Antipneumococcus Serum against Friedländer's Bacillus (K).

Friedländer's bacillus (K) (Type E).	Antipneumococcus sera.		Anti-Friedländer Serum E.	Virulence controls.
	Type I. 0.2 cc.	Type II. 0.2 cc.	Rabbit 84. 0.2 cc.	
0.1		D. 42	D. 18	
0.01		" 18	S.	
0.001	D. 18	S.	"	
0.0001	" 18	"	"	
0.00001	" 42	"	"	D. 21
0.000001	" 18			" 42
0.0000001				" 19

TABLE XIV.

Protective Action of Anti-Friedländer Sera against Pneumococcus Type II.

Pneumococcus Type II culture.	Virulence controls.	Anti-Friedländer serum.		Antipneumococcus serum Type II (Horse 91 A). 0.2 cc.
		Rabbit 84 D immunized with Strain E. 0.2 cc.	Rabbit 77 D immunized with Strain Sc. 0.2 cc.	
cc.				
0.2		D. 46	D. 20	D. 42
0.1		" 46	" 20	" 72
0.01		S.	" 20	S.
0.001		"	" 20	"
0.0001		"	" 26	"
0.00001	D. 36			
0.000001	" 46			
0.0000001	" 46			

länder's bacillus. Moreover, Table XIV brings out the fact that protective power against Type II pneumococcus infection is not possessed by the anti-Friedländer serum produced by Strain Sc, which both by agglutination and protection has been shown to belong to a type different from the effective Type E strains.

The further data presented in Tables XIV, XV, and XVI demon-
strate the comparable protective power of antipneumococcus Type II
and the anti-Friedländer E sera against infection with virulent Type

TABLE XV.

Protective Action of Anti-Friedländer Serum E against Pneumococcus Type II.

Pneumococcus Type II culture.	Immune sera.				Virulence controls.
	Anti-Friedländer E (Rabbit 84).		Antipneumococcus II (Horse 91 A).		
	Amount.	Result.	Amount.	Result.	
cc.	*cc.*		*cc.*		
0.2	0.2	D. 19	0.2	D. 24	
0.1	0.2	S.	0.2	" 48	
0.01	0.2	"	0.2	S.	
0.001	0.2	"	0.2	"	•
0.00001	0		0		D. 20
0.000001	0		0		" 20
0.0000001	0		0		" 36

0.0000001 cc. of this culture = 450 colonies.

TABLE XVI.

Comparative Protective Value of Anti-Friedländer and Antipneumococcus Serum against Pneumococcus Type II.

Culture Pneumococcus Type II.	ımmune sera.				Virulence control.
	Anti-Friedländer E (Rabbit 84).		Antipneumococcus Type II (Horse 91 A).		
	Amount.	Result.	Amount.	Result.	
cc.	*cc.*		*cc.*		
0.2	0.2	S.	0.2	D. 44	
0.1	0.2	"	0.2	" 20	
0.01	0.2	"	0.2	" 44	
0.001	0.2	"	0.2	S.	
0.0001	0.2	"	0.2	"	
0.00001	0		0		D. 26
0.000001	0		0		" 26
0.0000001	0		0		" 44

II pneumococci.   Indeed the protective potency of the anti-Fried-
länder serum in pneumococcus infection in two of the three experi-
ments is greater than, and in the third test, equal to that exhibited

by the Pneumococcus Type II immune serum itself. This is all the more striking since in the former case immune rabbit serum was used and in the latter the serum of a horse which had received more intensive immunization.

The peculiar "specificity" of the reciprocal protection of antisera of two kinds of bacteria so widely different in other biological characters is confirmatory of the immunological relationships of these organisms brought out by the cross-reactions of agglutination and precipitation.

DISCUSSION.

While comparison of the chemical properties of the two soluble specific substances isolated from Pneumococcus Type II and Friedländer's bacillus (Strain E) reveals many points of resemblance, differences are also found which are too great to be ignored. That the substances in reality are identical and that the observed differences depend only upon impurities present may possibly be the case, but the evidence so far obtained is entirely opposed to this assumption. It has been pointed out in the preceding paper (1) and in the papers dealing with the soluble specific substances of pneumococci (2, 5) that widely differing methods of preparation, calculated to remove different kinds of accompanying inert matter, have yielded strictly comparable products. This fact cannot be taken to indicate that the specific substances as at present isolated are pure chemical compounds, but it at least makes reasonable the assumption that in each instance a large proportion of the adventitious impurities has been eliminated. The view that the specific substance isolated from Pneumococcus Type II and that recovered from the Friedländer bacillus (Strain E) are not identical is further supported by certain of the serological findings, especially those which show that the absorption of agglutinins and precipitins is not reciprocal with the two organisms. If the fact that bacteria possess mutual absorptive capacity be accepted as the criterion of their antigenic identity then the failure of the organisms in question to exhibit this property may be taken as further evidence of the lack of identity of the substances involved.

However, granted a chemical difference between the two specific substances, it becomes necessary to account for their marked im-

munological similarity. In the absence of further evidence as to the structural relations of the two substances, which can only be obtained when large amounts of material become available, it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This essential similarity in molecular grouping would then determine the immunological similarity of the two substances.

In the case of Pneumococcus it has been shown that the polysaccharides by themselves are not antigenic, and it is believed that they become antigenic only when attached to some other substance, possibly the protein of the cell. The type-specific character of the antigenic response, however, is dependent almost entirely upon the nature of the polysaccharide and not upon the substance to which it is attached. Therefore, since the specific carbohydrate substance of the Friedländer bacillus (Strain E) and that of Type II pneumococcus possess in common similar chemical properties, the antigenic response to each may also be similar even though the proteins or other substances with which they are combined be quite dissimilar. A discussion of the actual number of antigens and antibodies present must be deferred until more facts are available (cf., however, in this connection, Landsteiner and van der Scheer (6)).

A striking and probably analogous example of common antigenic properties in substances of remote biological origin is furnished by the phenomenon of heterogenetic specificity originally described by Forssman (7). This investigator showed that following the injection of animal tissues of unrelated species common hemolytic antibodies for sheep corpuscles appear. Landsteiner (8) and Taniguchi (9) have shown that such heterogenetic antigens consist of two component parts, one a protein, the other probably a lipoid substance. Landsteiner and Simms (10) have found that the lipoid constituent, although itself practically devoid of antigenic properties, acquires true antigenicity when combined with protein, and that the antibodies thus induced react with the isolated lipoid fraction.

The fact that two biologically unrelated organisms, Pneumococcus Type II and Friedländer's bacillus (Strain E), possess certain similar serological and antigenic properties suggests that examples of heterogenetic specificity likewise occur among bacteria. In the case

of bacteria, however, the specific substance involved instead of being a lipoid appears to be a polysaccharide.   From the results reported in the present papers it further appears probable that when the analogous specific polysaccharides of otherwise totally unrelated micro-organisms correspond sufficiently in chemical constitution an immuno-logical correspondence also results.   This type of immunological correspondence in no way invalidates the systematic classification of bacteria based upon the more usual and general methods of species determination.   It is of greater immediate significance in connection with the study of problems dealing with bacteria as disease-producing agents than in the study of bacteria in their genetic relationships.

SUMMARY.

The chemical and immunological properties of the soluble specific substances of a strain of Friedländer's bacillus and Pneumococcus Type II are described and correlated, and the serological and anti-genic similarity of these biologically unrelated organisms is discussed as an example of heterogenetic specificity among bacteria.

BIBLIOGRAPHY.

1. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 701·
2. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 727·
3. Personal communication.
4. Krumwiede, C., Cooper, G., and Provost, D. J., *J. Immunol.*, 1925, x, 55.
5. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.   Heidel-berger, M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 301.
6. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xlii, 123.
7. Forssman, J., *Biochem. Z.*, 1911, xxxvii, 78.
8. Landsteiner, K., *Biochem. Z.*, 1921, cxix, 298.
9. Taniguchi, T., *J. Path. and Bact.*, 1921, xxiv, 253, 254.
10. Landsteiner, K., and Simms, H. S., *J. Exp. Med.*, 1923, xxxviii, 127.

THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

THIRD PAPER.

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In the preceding papers of this series (1) the significance of the so called "soluble specific substance" of Pneumococcus (2) was discussed and methods were given for the isolation of the specific substances of Types II and III pneumococcus. In the present communication refinements of these methods are described. With their aid it has been possible to obtain both of these substances free from nitrogen and possessing much greater activity when tested with the homologous immune sera than did the products previously isolated. Further data are presented concerning the nature of the polysaccharides with which these substances appear to be identified.

The isolation of a specific substance from Type I pneumococcus is also described, and it is shown that this substance, while apparently also a sugar derivative, contains nitrogen and differs also in other respects from the specific substances isolated from the other two fixed types of pneumococci.

EXPERIMENTAL.

1. The Soluble Specific Substance of Type I Pneumococcus.

The original method of purification of the soluble specific substance was modified after numerous trials to fit the properties of the Type I substance.

8 day cultures of Type I pneumococcus in meat infusion phosphate broth are concentrated in 21 liter lots on the water bath to a volume of 1.2 to 1.5 liters. The concentrate is precipitated with 1.4 volumes of alcohol and put through the

initial 3 layer separation as described in Paper I.   The middle layer[1] is dissolved
as well as possible in 200 to 300 cc. of water and the mixture is centrifuged and
the precipitate washed twice with small amounts of water.   The clear solution
and washings are kept on ice and combined with subsequent lots until 300 to 325
liters have been worked up.   After solution of the deposit of salts the total
concentrate, at a volume of about 4.5 to 5.0 liters, is treated with 1:1 hydro-
chloric acid until strongly acid to Congo red.   The resulting precipitate is
allowed to settle for several hours in the cold, after which as much of the super-
natant liquid as possible is siphoned off and the remainder cleared by centrifuga-
tion.   The precipitate is washed once with 0.01 normal hydrochloric acid and
again separated by repeated centrifugation at high speed.   The clear solution
and washings, at a volume of 5 to 6 liters, are now precipitated with 7 to 8 liters
of chilled alcohol and allowed to stand overnight in the cold.   A test portion of
the supernatant liquid, when neutralized and boiled down to small volume,
should give no immediate precipitate with Type I antiserum.   As much as
possible is siphoned off and the crude specific substance separated from the
remainder by centrifugation.   The precipitate is then washed in the centrifuge
bottles with 0.5 normal acetic acid, in which the isoelectric Type I substance is
insoluble, while most of the accompanying glycogen or erythrodextrin dissolves.
After several hours in the cold the mixture is centrifuged and the precipitate
taken up in water.   The specific substance is dissolved by addition of sodium
hydroxide until the mixture is faintly alkaline to litmus, the volume is adjusted
to about 700 cc., and the mixture is centrifuged at high speed until clear.   The
precipitate is washed with 50 cc. of water and again centrifuged.   To the solu-
tion and washings are added 20 gm. of sodium acetate, and when this is dis-
solved 400 cc. of alcohol are added, with vigorous stirring, and the mixture is
allowed to stand overnight in the cold.   In relatively concentrated solution and
in the presence of sufficient salts the Type I specific substance is quantitatively
precipitated by 0.5 volume of alcohol in the cold.   The use of sodium acetate
is advantageous as it is not thrown out by alcohol at the concentrations used.
The precipitate is centrifuged off, water is added up to a volume of about 500
cc., the mixture is made slightly alkaline again if necessary, and is finally centri-
fuged to remove any insoluble material.   The solution is then stirred mechan-
ically and the specific substance is rendered insoluble by the careful addition of
16 cc. of glacial acetic acid.   After several hours in the cold it is centrifuged off
and redissolved with the aid of sodium hydroxide as before, except that the
volume should now be about 400 cc.   15 gm. of sodium acetate are added, and
the specific substance is precipitated in the cold with 200 cc. of alcohol.   After
having stood overnight it is centrifuged off and redissolved in enough water to
bring the volume to about 250 cc., using enough sodium hydroxide to make

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[1] Centrifuged precipitates containing the soluble specific substances are
usually compact and gummy, and care must be taken to smooth out all lumps
when the deposit is redissolved.

the reaction slightly alkaline. The solution is chilled, stirred mechanically, and treated with an equal volume of barium hydroxide solution in equilibrium with the crystalline phase at about 50°. Most of the specific substance separates at once, and the mixture may be centrifuged after several hours in the ice box. Under optimal conditions the precipitation is complete, but occasionally up to about 15 per cent of the specific substance may remain in solution. If this is to be recovered it is best worked up separately, as coloring matter and other impurities are eliminated with it. The barium hydroxide precipitate is taken up in about 400 cc. of water and treated with 5 normal sulfuric acid until, after all lumps are smoothed out, the reaction remains acid to Congo red. The mixture is then centrifuged, and the precipitate of barium sulfate washed twice with very dilute sulfuric acid. The supernatant solutions are neutralized, and if opalescent, may be passed first through a Berkefeld V candle and then through the W grade. The solution is concentrated *in vacuo* to about 200 cc., made faintly alkaline with sodium hydroxide, and precipitated in the cold with 100 cc. of alcohol after the addition of 10 gm. of sodium acetate. The next day the precipitate is collected in the usual way, redissolved in water, and at a volume of about 200 cc. is again thrown out at the isoelectric point with 7 cc. of glacial acetic acid, using the same precautions as before. The precipitate is taken up in a little water and redissolved by addition of 1:1 hydrochloric acid. The volume is made up to about 125 cc. with water and hydrochloric acid, and the specific substance is then precipitated as the hydrochloride by the addition, with mechanical stirring, of 250 cc. of chilled redistilled alcohol. The centrifuged product is redissolved and reprecipitated in the same way and is then taken up in as small a volume as possible of cold water. The extremely viscous solution is then dialyzed in collodion bags in the ice box against successive changes of distilled water. The Type I specific substance is a weak base, and as the excess of hydrochloric acid diffuses out the hydrochloride is hydrolyzed and the isoelectric substance precipitates. Completion of the process and removal of the last traces of chlorine ion are accelerated by mixing the contents of the bags each time the water is changed. The mixture is finally centrifuged, the solution, which contains a little active material, is added to the next preparation, and the precipitate is washed with acetone, filtered on a hardened paper in a Büchner funnel, and dried. The yield should be from 2 to 3 gm.

The Type I specific substance obtained by this method contains 5 per cent of nitrogen and is often ash-free and colorless, although some preparations are still grayish in color and contain a little ash. In its isoelectric form the product is very sparingly soluble, but may be dissolved with the aid either of alkali or mineral acid. Concentrated solutions are extremely viscous. The specific optical rotation, $+300°$, is about the same on either side of the isoelectric point, which is

in the vicinity of pH 4. When boiled with a sufficient excess of mineral acid the substance is slowly hydrolyzed with formation of reducing sugars, which it has not yet been possible to identify fully. Reducing sugars also appear when the substance is treated with nitrous acid in the cold. One-half of the nitrogen is evolved at the same time, with simultaneous disappearance of the specific reaction. Since the specific substances of Types II and III pneumococcus are unaffected by nitrous acid under these conditions, it would appear that this half of the nitrogen, at least, is an integral part of the specific substance and is either linked to the reducing group of a sugar derivative, as Karrer believes is the case with the polyglucosamines (3), or else is so placed in the complex molecule that its removal causes some other type of scission. About one-half of the remaining nitrogen in the substance is given off within 15 minutes when the reaction mixture, freed from nitrous acid, is made alkaline and distilled with steam. The substance contains no sulfur or phosphorus.

When a concentrated aqueous solution of the hydrochloride of the specific substance is carefully treated with 1:1 hydrochloric acid a point is reached, at an acidity of 1.3 normal, at which the substance again precipitates, redissolving again if still more acid is added. Whether this indicates lactam formation, or some other reversible intramolecular change, is not yet clear.

½ per cent solutions, prepared by dissolving the specific substance in dilute acid and bringing it back past the isoelectric point with dilute sodium hydroxide, give precipitates with solutions of the following reagents: barium hydroxide, copper sulfate, silver nitrate, neutral and basic lead acetates, uranyl nitrate, and phosphotungstic acid. There is no color developed with iodine-potassium iodide solution, nor does the unhydrolyzed substance show reduction with Fehling's solution. The biuret, tannic acid, xanthroproteic, and ninhydrin tests are negative. With Millon's reagent a jelly is formed in the cold, but the precipitate redissolves on heating and no color is developed. Potassium permanganate is not immediately reduced.

After hydrolysis with nitric acid and subsequent oxidation mucic acid was obtained.

1.5 gm. of air-dry Preparation 39 were dissolved in 200 cc. of hot 1.4 normal nitric acid and boiled under a reflux condenser for 4 hours. The resulting green-

ish yellow solution was concentrated *in vacuo* to about 10 cc., and 4 cc. of concentrated nitric acid were added. The next day the mixture was boiled for a few moments and then stirred on a large clock-glass over boiling water until dry. The process was repeated with a few cc. of 1:1 nitric acid, after which the dry, white residue was taken up in acetone and allowed to stand overnight. The crystalline residue was filtered off, washed with a little acetone, and taken up in 5 cc. of normal ammonium hydroxide. The insoluble portion, apparently calcium oxalate, was discarded. Addition of 0.3 cc. of concentrated nitric acid to the filtrate resulted in the deposition of 0.21 gm. of mucic acid melting and decomposing at 216° with preliminary softening and blackening. The acetone mother liquors above were concentrated to dryness and taken up in water, when a second fraction of mucic acid was left behind. This was purified in the same way, yielding 0.07 gm., melting and decomposing at 215°. For analysis the two fractions were combined and recrystallized from water.

0.1008 gm., anhydrous, ash-free substance gave 0.1253 gm. CO_2 and 0.0442 gm. H_2O.

Calculated for $C_6H_{10}O_8$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 33.9 per cent; H, 4.9 per cent.

From the aqueous mother liquors from which the second crop of mucic acid was obtained was isolated an acid potassium salt in a yield of 0.191 gm. and containing 22.3 per cent of potassium.

A repetition of the oxidation in the cold with 1 gm. of Preparation 41 gave after 1 week a deposit of mucic acid weighing 0.115 gm. after two recrystallizations.

Since the Type I specific substance gives a positive test for glucuronic acid with naphthoresorcinol it is possible that galacturonic acid is present as a part of the molecule, for this would yield mucic acid on oxidation. That the mucic acid does not arise from oxidation of galactose itself is indicated by the difficulty with which an osazone is formed from the products of hydrolysis, although crystallization of the osazone formed might also be hindered by the complexity of the reaction mixture.

While the Type I specific substance as at present obtained probably does not represent a single, chemically pure compound, it would nevertheless seem as if the major portion of the impurities had been removed. As will be seen from Table I, in which the figures given are calculated to the ash-free basis, the total nitrogen, the nitrogen and reducing sugars liberated on treatment with nitrous acid, and the optical rotation are remarkably constant in the majority of instances, although the preparations listed were isolated in different ways.

29B was prepared from Type I pneumococci themselves, instead of the autolyzed culture fluid, by solution in diluted bile, removal of the "nucleoprotein" with acetic acid, and adsorption of the specific substance on alumina, as described in the case of Type II pneumococcus (1).

In 36 and 37 the use of barium hydroxide was omitted, necessitating a more laborious fractionation. 37A was prepared from 37 by adsorption on a particularly active form of alumina (Alumina A) prepared according to the directions of Willstätter and Kraut (4).

41B$_2$ was obtained from 41 by interaction with neutralized formalin in slightly alkaline solution. Discharge of the phenolphthalein color indicated that the specific substance combined with the aldehyde. However, fractional precipitation of the resulting substance with alcohol (B$_2$ being the second fraction) failed to yield a product with different properties. That the combination with formaldehyde was a labile one was indicated by the fact that the product recovered, when once more treated as before with formalin, again showed an increase in the acid reaction, indicating that any formaldehyde combined with the specific substance had been eliminated in the process of isolation (addition of cold dilute hydrochloric acid and precipitation with alcohol).

43A was obtained by digestion of the product of the third alcoholic precipitation (see method) with an amount of ice-cold water sufficient to dissolve only a portion of the specific substance. The mixture had a total volume of 300 cc. in this case, and the specific substance was reprecipitated with alcohol and then purified with the aid of barium hydroxide. The residue from the A fraction was worked up in the usual way as 43B, while the part of it not precipitated by barium hydroxide, but separating when the alkaline mother liquors were acidified with acetic acid, is represented by 43C. It will be seen from Table I that each of these fractions gave the same analytical values, and the physical properties corresponded equally closely.

Finally, 44A, after precipitation with barium hydroxide, removal of the barium, and precipitation with alcohol, was purified twice over the sparingly soluble hydrochloride mentioned above. It was hoped that precipitation in this way by hydrochloric acid without the assistance of alcohol would result in the removal of nitrogenous or carbo-

TABLE I.

Preparation No.	Specific rotation.	Total N.	Amino N.	Reducing sugars on acid hydrolysis.*	Reducing sugars by HNO₃.	C	H	Ash.	Precipitation with anti-pneumococcus serum.†

Type I pneumococcus.

		per cent	per cent	per cent	per cent	per cent	per cent	per cent	
29B	+301°	4.8	2.7	31.8		43.3	5.8		1:4,000,000
36	+304.5°	4.6‡	2.4	31.6				1.5	1:6,000,000
37	+295.5°	5.1	2.6	27.2				0.7	1:6,000,000
37A	+287°	4.1‡	2.5		31.7			7.9	1:6,000,000
38A	+310°	5.0	2.6	28.6	28.5			1.8	1:6,000,000
39	+304°	4.4‡	2.5		27.1			1.6	1:6,000,000
41	+303°	5.0	2.6		26.0			0.0	1:8,000,000
41B₂	+279°	4.4‡	2.5		23.9				
43A	+303°	5.0	2.5		23.8			1.2	1:6,000,000
43B	+300°	4.9	2.5	28.4	23.5			0.0	1:6,000,000
43C	+300°	5.0	2.6		28.0			0.0	1:6,000,000
44A	+300°	5.0	2.6		23.5	43.3	5.5	0.5	1:8,000,000

Type II pneumococcus.

					Acid equivalent.				
25	+63.2°	0.18		80.3				1.9	1:3,000,000
25A	+70.2°	0.0		68.4	1302	45.8	6.4	0.35	1:6,000,000
25B	+56.7°	0.0			946				1:2,000,000
25C	+72.2°	0.12		67.6	1190				1:5,000,000
26A	+75.2°	0.0		69.1	1258			1.6	1:6,000,000
26A₁	+72.8°	0.0		68.2	1252			0.0	1:5,000,000
26B	+54.4°	0.0		56.0	1105				

Type III pneumococcus.

30	−37.3°			73.0	330				1:6,000,000
30I	−30.9°	0.0		73.3	347				1:5,000,000
30A	−35.1°	0.0		71.0	339			0.0	1:6,000,000
33	−30.5°	0.0		73.0	343			0.0	
33I	−32.5°	0.0		74.5	341			0.0	
33II	−34.0°	0.0		75.5	340	42.7	5.3		
33B III	−30.8°	0.0		72.5	358			0.0	1:6,000,000

* Calculated as glucose.

† 2 hours at 37° and overnight in the cold.

‡ Micro Kjeldahl determinations using aeration method for collecting NH₃. Results about 10 per cent low.   In the other cases Pregl's method was used.

hydrate material that would otherwise be precipitated by the alcohol customarily added.   However, this preparation checked closely with the others in optical rotation, carbon, hydrogen, and nitrogen, and in the other quantitative and qualitative tests used.

2. Type II Pneumococcus.

A. Further Purification of the Type II Soluble Specific Substance.

The specific substance from 312 liters of broth culture was isolated according to the method given in Paper II.   After the third precipitation with ammonium sulfate the deposited material was redissolved in hot water, run through a Berkefeld W candle to remove opalescence, and concentrated to 150 cc.   The solution was cooled to 0°, acidified with 35 cc. of 1:1 hydrochloric acid, stirred mechanically, and precipitated by the addition of 350 cc. of chilled redistilled alcohol.   After 2 hours in the ice box the precipitate was centrifuged off in the cold and redissolved in 75 cc. of cold water.   The precipitation process was then repeated with 18 cc. of 1:1 hydrochloric acid and 300 cc. of alcohol.   The precipitate, after centrifuging, was redissolved in 100 cc. of water, rinsed into a collodion bag with 5 cc. of normal hydrochloric acid, and dialyzed in the cold against distilled water until free from chlorine ion.   The solution was concentrated to 50 cc. *in vacuo* and stirred into 600 cc. of redistilled acetone.   After having stood overnight the precipitate was collected on hardened paper, washed with redistilled acetone, and dried.   The yield was 3.3 gm. in the case of Preparation 26A, and 2.4 gm. for 25A.

The supernatant from the initial acid-alcohol precipitation still reacted strongly with Type II antiserum and was accordingly precipitated with 0.5 volume of ether.   The material deposited was dissolved in 20 cc. of water and, after addition of a little hydrochloric acid, was dialyzed as in the previous instance, concentrated to 20 cc., and poured into 12 volumes of acetone.   0.6 gm. was recovered (26B) in one case and 0.5 in another (25B).

In an attempt to push the purification further a portion of 26A was again put through the acid-alcohol precipitation process.   The product, 26A$_1$, gave, however, the same analytical values as that from which it was derived.

From a comparison of Preparation 25, Table I, in which these additional steps in the purification process were omitted, with 25A, 26A, and 26A$_1$, it will be seen that the remaining nitrogen in the specific substance has been eliminated, all but traces of ash have been removed, a fraction (25B and 26B) has been separated containing impurities of lower optical rotation, and the highest dilution at which the substance reacts with its homologous serum has been increased. In order to reduce the analytical error to a minimum micro Kjeldahl determinations were run on 25 mg. of substance in the case of 26A.

B. *Confirmation of Glucose as the Chief Sugar Constituent of the Type II Specific Substance.*

In the previous papers it was shown that glucosazone could be obtained from the hydrolysis products of the Type II soluble specific substance. Although the hydrolyzed material showed a specific rotation of about $+55°$, indicating that the resulting hexose was probably glucose, the possibility that the sugar might have been mannose or fructose was by no means excluded, owing to the possible presence of other optically active substances.

In order definitely to determine this point, a portion of the specific substance was hydrolyzed and subsequently oxidized.

0.5 gm. of Preparation 25A was dissolved in 25 cc. .of water and boiled for 5 hours under a reflux condenser with an equal volume of normal nitric acid. The solution was evaporated *in vacuo* to 3 to 4 cc., treated with 2 cc. of concentrated nitric acid, and allowed to stand at room temperature overnight. The mixture was then boiled for 2.5 minutes over a flame, and poured on to a large watch-glass, stirred, and quickly evaporated over a boiling water bath. A thick paste was obtained and this was twice evaporated with water to expel the last traces of nitric acid, dissolved in 2 cc. of water, made strongly alkaline with 40 per cent potassium hydroxide solution, and reacidified with glacial acetic acid. The mixture was cooled in ice water, and after 24 hours in the cold the crystals of potassium acid saccharate which had separated were filtered off. 0.1 gm. was recovered. This was recrystallized from 1.5 cc. of water, yielding 0.062 gm. of the purified salt.

0.0602 gm. substance gave 0.0210 gm. K_2SO_4.

Calculated for $KOOC(CHOH)_4COOH$: K, 15.75 per cent. Found: 15.65 per cent.

The isolation of potassium acid saccharate through the oxidation of the ·hydrolytic products of the Type II soluble specific substance, together with the isolation of glucosazone previously reported, leaves little doubt that the units from which the polysaccharide is built up are those of glucose.

C. *Preparation of a Triacetate of the Type II Soluble Specific Substance.*

If the true soluble specific substance were actually a polysaccharide it should be possible to convert it into an acetyl derivative just as the celluloses and starches may be acetylated. On the other hand,

if the polysaccharide present were an impurity the altered solubilities of an acetyl derivative might be expected to facilitate its removal from the actual specific substance.

0.5 gm. of Preparation 25A was accordingly dried to constant weight *in vacuo* and shaken overnight at room temperature with a mixture of 2.5 cc. of dry acetic anhydride and 6 cc. of dry pyridine.   Since no reaction appeared to have taken place the tightly stoppered bottle containing the mixture was heated in a water oven for 24 hours.   The resulting suspension, which had darkened somewhat, was poured into ice water but most of the solid failed to dissolve.   It was therefore filtered off, macerated with water until the washings failed to give a specific test with Type II antiserum, and shaken with 200 cc. of water and a few glass beads until it had disintegrated into a very fine powder.   After centrifugation the supernatant fluid gave only a weak test with immune serum.   The residue was next washed with 50 cc. of methyl alcohol, which removed most of the coloring matter, and was dried *in vacuo*.   0.7 gm. of an amorphous, fluffy, tan powder was recovered.

0.1009 gm. substance gave 0.1864 gm. CO_2 and 0.0503 gm. H_2O.

Calculated for $[C_6H_7O_5(OCOCH_3)_3]_x$: C, 49.95 per cent; H, 5.60 per cent. Found: C, 50.4 per cent; H, 5.6 per cent.

0.1084, 0.1190 gm. substance hydrolyzed with 0.5 N NaOH, neutralized 9.70, 10.90 cc. 0.1 N NaOH respectively.   Calculated: 11.30, 12.40.

The triacetate was found to be practically insoluble in water and the common organic solvents.   It dissolved, however, when warmed with dilute sodium hydroxide, the neutralized solution giving a heavy precipitate with antiserum.   On account of the extreme insolubility of the triacetate it was difficult to determine definitely whether or not it was specific in itself.   However, the large yield and the fact that it again yields a specifically reacting substance on hydrolysis showed that no separation of the polysaccharide from the actual specific substance had been effected.

D. Recovery of the Type II Specific Substance from Its Precipitate with Homologous Antibody.

In Paper II it was shown that the Type II specific substance could be recovered from its precipitate with immune serum.   Owing to the difficulty met with in separating the polysaccharide from large amounts of serum protein it seemed advisable to repeat this experiment, using instead of the serum an antibody solution purified essentially according to Felton (5).

1.5 liters of Type II antipneumococcus serum were gradually added with stirring to 28.5 liters of well chilled N/100 acetic acid-sodium acetate buffer at pH 5. After having stood overnight the precipitate was centrifuged off, dissolved in 750 cc. of 0.85 per cent salt solution, centrifuged once more, and again added to 19 volumes of the same buffer solution. The resulting precipitate, which contained a large proportion of the antibodies in the original serum, was finally collected as before and dissolved in 1.5 liters of saline.

To this solution was slowly added 0.2 gm. of Preparation 25A dissolved in 500 cc. of saline. After 2 hours in the incubator at 37° and standing in the ice box overnight the precipitate was centrifuged off and was washed with 150 cc. of saline, centrifuged again, and suspended in 150 cc. of a citrate-phosphate-borate buffer solution prepared according to Northrop (6). The mixture was digested at 37° with 1 gm. of trypsin added in small portions at intervals until a clear solution was obtained. This was then treated with 2 volumes of alcohol and the resulting precipitate was suspended in water and treated with dilute sodium hydroxide solution until it just dissolved. The solution was heated to boiling and neutralized with dilute acetic acid, causing the separation of a coagulum. The supernatant liquid containing the specific substance was evaporated to complete dryness and taken up in boiling water. A small amount of insoluble protein material was centrifuged off and the supernatant liquid was dialyzed until free from salts and repeatedly evaporated and extracted with water. With each evaporation a small amount of insoluble protein material was eliminated. The extract was finally saturated with ammonium sulfate, whereby a heavy yellow protein precipitate separated. This was centrifuged off, redissolved in a little water, reprecipitated with ammonium sulfate, and again centrifuged. The supernatant liquid containing the soluble substance was again dialyzed until free from sulfate. The solution was then concentrated to 5 cc. and precipitated with 10 cc. of alcohol and a few crystals of sodium acetate. The inactive supernatant liquid was discarded and the precipitate was twice reprecipitated by alcohol from a volume of 5 cc. The substance was finally precipitated from the same volume at 0° by the addition of 1.5 cc. of 1:1 hydrochloric acid and 2 volumes of alcohol. It was then dissolved in a few cc. of water and added to a large excess of acetone. The yield was 0.12 gm.

The recovered specific substance (25C) failed to give the biuret reaction and contained only 0.12 per cent of nitrogen, whereas in the original experiment with serum the nitrogen content was 1.0 per cent. In its other properties it agreed well with the highly purified material used to precipitate the antibody solution.

3. *The Soluble Specific Substance of Type III Pneumococcus.*
A. Further Attempts at Purification.

A preparation of the Type III specific substance which had been twice purified by precipitation with hydrochloric acid (see Paper II) was reprecipitated three times in this manner in order to determine whether any additional impurities could be eliminated. However, the preparations, 33, 33I, and 33II checked well in analytical values with each other and with previous preparations, except that no nitrogen was found, although in the case of 33I 35.5 mg. of substance were used for each micro Kjeldahl determination.

Since it had been found that the Type III substance gave a precipitate when barium hydroxide in excess was added, an additional purification of the substance was attempted over the barium salt.

0.5 gm. of Preparation 33 was finely pulverized and suspended in 25 cc. of water. The mixture was heated to boiling under a reflux condenser and to it was added enough 0.2 normal barium hydroxide solution to keep the reaction just alkaline to phenolphthalein. The solution was centrifuged from a few particles which had failed to dissolve, and the supernatant liquid, which contained practically all of the substance as a soluble barium salt, was treated with a slight excess of saturated barium hydroxide solution. After the resulting heavy flocculent precipitate was centrifuged off the supernatant liquid gave only a slight precipitate with immune serum. The barium salt was washed once with water containing a small amount of barium hydroxide and was then dissolved in about 350 cc. of hot water and treated cautiously with sulfuric acid until the supernatant liquid showed neither barium nor sulfate ions. The barium sulfate was centrifuged off and the supernatant liquid concentrated *in vacuo* to small bulk and dialyzed in a collodion bag. After further concentration *in vacuo* the solution was added to 10 volumes of redistilled acetone. The precipitate was sucked dry on a filter, washed with acetone, and dried *in vacuo* over phosphorus pentoxide until constant in weight, a process which rendered the recovered Type III acid insoluble. The yield was 0.43 gm.

A comparison of the properties of Product 33B III with those of 33 (Table I) indicates that precipitation of the Type III acid as the barium salt does not effect a fractionation.

An attempt was also made as in the case of Type I to remove possible impurities by adsorption of the specific substance on the highly reactive Type A alumina prepared according to the method of Willstätter and Kraut (4).

As a result of preliminary experiments it was found that at a pH below 6.0 1 gm. of gel (calculated as Al_2O_3) adsorbed approximately 0.35 gm. of polysaccharide. At a pH higher than 7.0 the adsorption of the Type III substance did not reach completion.

0.5 gm. of Preparation 30 was dissolved in the equivalent amount of N/14 sodium hydroxide. To the solution was added a suspension of 1.5 gm. of alumina and the volume was made up to 750 cc. The pH was adjusted with N/5 hydrochloric acid to 5.0 and the suspension was shaken for 1 hour, after which the supernatant fluid gave a negative precipitin test. The mixture was centrifuged and the precipitate washed once with water. It was then extracted twice with N/5 sodium carbonate solution and the extract, after neutralization, was concentrated *in vacuo*, dialyzed in parchment, again concentrated *in vacuo*, and dialyzed in collodion until free from chloride ion. The solution was then further concentrated *in vacuo* and precipitated from a volume of 15 cc. by 5 cc. of 1:1 hydrochloric acid and 40 cc. of redistilled alcohol at 0°. The specific acid was washed free from chlorides with 50 per cent alcohol and finally with acetone. 0.36 gm. of dry material was recovered.

A comparison of Product 30A with the starting material shows that adsorption of the specific substance on alumina did not result in the separation of any significant amount of impurity, a result also given by the other methods of purification attempted.

The variations shown by the optical rotation of the Type III substances listed in Table I may perhaps be explained by the fact that in each instance the insoluble acid was dissolved in a slight excess of dilute alkali. It was found that alkaline solutions of the substance suffered a slow decrease in optical rotation, followed by a slow return to the original value after acidification. For instance, in one case a portion of Preparation 33II, with an initial $[\alpha]_D$ of $-34.1°$ showed a drop in 0.7 per cent sodium hydroxide solution to a value of $-21.2°$, this returning to $-36.0°$ after acidification.

B. Hydrolytic Products of the Type III Specific Acid.

As a preliminary step in the study of the products of hydrolysis, the relation between the specificity, the time of hydrolysis, the percentage of reducing sugars, and the optical rotation was studied.

0.0836 gm. of dried Preparation 30I, of which the $[\alpha]_D$ was $-30.9°$, was dissolved in 0.55 cc. of concentrated sulfuric acid at $-10°$ and allowed to stand at this temperature for 1 hour. The viscous solution was then diluted to 21 cc.

to give an approximately normal solution of acid, and, before hydrolysis on boiling, its optical rotation, reducing power, and specificity were tested. The values observed are to be found in the following table.

Period of boiling.	$[\alpha]_D$	Reducing sugars (calculated as glucose).	Reaction with Type III antipneumococcus serum.
min.		*per cent*	
0	$-15.9°$	0.0	++
140	$+8.8°$	54.8	±
180	$+15.4°$	61.0	−
300	$+22.6°$	69.4	−
360	$+23.4°$	65.5	−

It is thus evident that in boiling normal sulfuric acid the specific reaction of the Type III acid persists up to the 3rd hour, and that the maximum reducing power is attained in about 5 hours.

6 gm. of Preparation 30I with a water content of 7.6 per cent, were finely pulverized and slowly added to a mixture of 30 gm. of concentrated sulfuric acid and 10 cc. of water at 0°. The acid was stirred slowly with a turbine and after a short time 10 cc. more of the sulfuric acid mixture were added. After 2 hours only a few lumps remained and the mixture was allowed to stand in the ice box overnight. It was then poured into 800 cc. of water and boiled under a reflux condenser for 5 hours, at which time the reducing power showed a constant value. The sulfuric acid was removed quantitatively with barium hydroxide. The barium sulfate was centrifuged off, and the liquid concentrated *in vacuo* to 100 cc., boiled with calcium-free Norite, and filtered. The colorless filtrate was treated with an excess of basic lead acetate and centrifuged. The precipitate was washed once with water containing a little basic lead acetate, three times with alcohol, and was then filtered off, washed with acetone, and dried. 10.7 gm. of the lead salt were recovered in this way.

The supernatant from the basic lead acetate precipitate contained a strongly reducing substance (0.96 gm. calculated as glucose) and was accordingly treated with hydrogen sulfide to remove lead, filtered, and concentrated, finally, *in vacuo* to dryness in a desiccator. After a few days crystals could be seen with a hand lens but on account of the difficulty in isolating them in appreciable quantities the whole was treated with 6 cc. of 1:1 nitric acid and oxidized as described previously in this paper.

0.15 gm. of recrystallized potassium acid saccharate was readily obtained.

0.0804 gm. substance gave 0.0282 gm. K_2SO_4.

Calculated for $COOK(CHOH)_4COOH$:. K, 15.75 per cent. Found: K, 15.73 per cent.

A similar sugar fraction from the hydrolysis of 4.0 gm. of Type III acid, containing 0.6 gm. of sugar calculated as glucose was de-leaded with hydrogen sul-

fide and treated in a volume of 50 cc. with 1.3 gm. of phenylhydrazine dissolved in 3 cc. of 50 per cent acetic acid.   After warming for 1 hour on the water bath the copious deposit of yellow crystals was filtered off, washed with 10 cc. of methyl alcohol to remove tar, filtered, and dried.   0.40 gm. of osazone was obtained, melting and decomposing at 203–204°.   A solution of 0.0760 gm. in 7.6 cc. of pyridine-alcohol mixture showed, in a 0.5 dm. tube, a rotation of −0.33°.   After 2 days the reading had decreased to −0.12°, mutarotation in this direction being a characteristic of glucosazone (7).   Recrystallized for analysis, the osazone decomposed at 206–210°.

Calculated for glucosazone, $C_{18}H_{22}O_4N_4$: N, 15.45 per cent.   Found: 15.42 per cent.

The salt formed as described above by the addition of basic lead acetate to the hydrolysis product was suspended in about 300 cc. of water and treated with hydrogen sulfide.   The lead sulfide was removed and the filtrate concentrated *in vacuo* to small volume, boiled with Norite, filtered, and the filtrate evaporated to complete dryness over phosphorus pentoxide.   3.1 gm. of a white glassy mass remained.   It was readily soluble in water, difficultly soluble in alcohol, and gave a strong glucuronic acid test with naphthoresorcinol.   However, its non-crystalline nature, its insolubility in alcohol, and the difficulty of isolating from it a bromophenylosazone did not support its being glucuronic acid itself.   Moreover its $[\alpha]_D$ was +12.3° (0.3372 gm. in 20 cc. of water), whereas the specific rotation of glucuronic acid is +19.1°.   It also had a reducing power of only 48.0 per cent, calculated as glucose.   This value could be increased about 10 per cent by boiling with 0.5 normal hydrochloric acid, while boiling with hydrobromic acid of the same strength increased the reducing power about 15 per cent and concentrated hydrochloric acid in the cold caused an increase of only 7 to 8 per cent.   Saccharic acid could not be isolated from the product by oxidation with bromine.   The reducing group was also readily oxidized by nitric acid, but the product again showed remarkable stability toward further hydrolysis.

It is thus quite certain that the second component of the hydrolysis products of the Type III acid is not glucuronic acid itself, although there is evidence that it consists of glucuronic acid combined with some other substance, perhaps a hexose derivative.   It is hoped to clear up this point when more material becomes available.

As to the portion of the hydrolysis products not precipitated by basic lead acetate, however, there can be no doubt that the principal substance present is glucose, owing to the high yield of glucosazone obtained, and to the isolation of acid potassium saccharate on oxidation of the sugar.

C. Conversion of the Insoluble Type III Acid into a Soluble Form.

When the insoluble form of the Type III acid, obtained in the usual way, is boiled with water it gradually enters into solution.

0.1 gm. of Preparation 30I was shaken for 1 hour with 200 cc. of water at 50°. Since only a swelling of the granules of the specific substance occurred, the mixture was diluted to a volume of 500 cc. and boiled under a reflux condenser for 1½ hours. The resulting clear solution was filtered as a precaution and concentrated, finally, in a vacuum desiccator until the volume was 5 cc. The solution, which was still clear, was thrown into 100 cc. of acetone and filtered. The precipitate was washed with acetone and absolute alcohol and was dried at room temperature over sulfuric acid at atmospheric pressure until a constant weight of 0.09 gm. was reached. The substance thus recovered was a white amorphous powder soluble in water, but after it was again dried *in vacuo* it gradually passed into the insoluble derivative. $[\alpha]_D$ of the soluble form was $-31.9°$, and the acid equivalent (352) was in agreement with the values obtained by solution of the insoluble form in dilute alkali and subsequent neutralization.

Whether the reversible equilibrium between the soluble and insoluble forms is one involving simple hydration and dehydration, or one depending on the opening and closing of a lactone or anhydride grouping cannot be decided with the evidence at present available.

DISCUSSION.

The following data are given in order to summarize briefly the properties of the soluble specific substances of the three fixed types of Pneumococcus, in the state of purity thus far attained.

The soluble specific substance of Type II pneumococcus is apparently a weakly acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its acid equivalent is about 1250 and the specific optical rotation is about $+74°$. It is not precipitated by barium hydroxide or heavy metal salts with the exception of basic lead acetate and uranyl compounds. It reacts at a dilution of 1:5,000,000 with Type II antipneumococcus serum but does not precipitate Type I and Type III antisera at a concentration of 1:400. The substance is converted by acetic anhydride and pyridine into a very sparingly soluble triacetyl derivative.

The Type III soluble substance, while also isolated as a nitrogen-

free polysaccharide, is a strong acid with an acid equivalent of about 340, and is made up not only of glucose units but also those of either glucuronic acid or a derivative of this acid.   It rotates the plane of polarized light about 33° to the left.   It is precipitated by barium hydroxide in excess and by heavy metal salts, and is also rendered insoluble by the addition of strong hydrochloric acid.   In as high a dilution as 1:6,000,000 it still reacts with Type III antipneumococcus serum.

The Type I soluble specific substance also appears to be a sugar derivative, but differs from the other two substances in its lower percentage of sugar liberated on hydrolysis and in containing nitrogen as an apparently essential component.   It rotates the plane of polarized light about 300° to the right, is a strong acid and a weak base, and is very sparingly soluble at its isoelectric point, which lies at about pH 4.   In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests.   One-half of the nitrogen is liberated on treatment with nitrous acid and reducing sugars appear at the same time, while the specific reaction vanishes.   Under the same conditions the Type II and Type III substances are unaffected by nitrous acid.   The substance gives the color reaction for glucuronic acid with naphthoresorcinol, but yields mucic acid on oxidation, indicating a relationship to galactose.   Since the carbon and hydrogen contents of the substance are close to the theoretical values for polysaccharides it appears possible that in it a nitrogenous sugar derivative is linked to galacturonic acid through the reducing group of the latter.   Further evidence on this point will be sought.   The Type I substance is precipitated by barium hydroxide in excess, by heavy metal salts, and by phosphotungstic acid.   In the specific precipitin reaction with homologous Type I antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000 while at a concentration of 1:400 it gives a faint cloud with Type III antiserum.

The three polysaccharides contain no sulfur or phosphorus and differ from the starch-glycogen group of carbohydrates in giving no color with iodine and in their resistance to the ordinary carbohydrate-splitting enzymes.

It would be, of course, idle to assume that in their present state of purity, each of the specific substances represents a definite chemical

compound. However, in the case of the three fixed types of Pneumococcus three totally distinct substances have been isolated from cultures grown in the same medium. Successive preparations of the specific substance have in each instance been quite uniform regardless of the widely different methods employed in the process of purification. Moreover, substances reactive to the same degree with homologous antisera have been derived both from the microorganisms themselves and from autolyzed broth cultures.

It is thought that these and other considerations based on the data presented warrant the belief that the three polysaccharides isolated represent the actual specific substances, stripped of at least a large portion of accompanying impurities, and that they do not merely represent inert material carrying an extremely minute amount of the true specific compounds.

If this be accepted it may be concluded that the soluble specific substance of each of the three fixed types of Pneumococcus is a distinct chemical substance, differing in many striking particulars from the corresponding product elaborated by the other two types, but having in common the properties of polysaccharide structure and of resistance to enzyme action. Each substance breaks down on hydrolysis into reducing sugars, a part of which, at least, is peculiar to itself. The Type I substance differs sharply from the other two in containing nitrogen and in possessing basic as well as acid properties, while of the other two substances, the Type II is a dextrorotatory weak acid and the Type III a levorotatory strong acid. Especially striking is the occurrence of specific substances of such widely differing properties in microorganisms as closely related as the three fixed types of Pneumococcus.

The immunological significance of the specific substances has been discussed by the writers in a recent paper (8) and will therefore not be touched upon in the present communication.

Many of the questions raised in the course of the work are still under investigation and the specific substances of other microorganisms are being studied.

SUMMARY.

1. Refinements in the methods for the isolation of the soluble specific substances of Types II and III pneumococcus are described.

These improvements have resulted in the isolation of the end-products in a form free from nitrogen and of enhanced activity with immune serum.

2. The soluble specific substance of Type I pneumococcus is described and shown to differ sharply from the corresponding substances of the other two types, each of which, in turn, differs from the other.

3. Progress is reported on the identification of the sugar units from which the polysaccharides are built up.

4. The evidence so far accumulated is believed to favor strongly the view that the polysaccharides isolated are the actual specific substances of Pneumococcus.

In conclusion the writers wish to express their gratitude to Dr. P. A. Levene, for his ready counsel and assistance, and to Dr. W. A. Jacobs for his help as well.

BIBLIOGRAPHY.

1. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
2. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
3. Karrer, P., Schnider, O., and Smirnoff, A. P., *Helv. Chim. Acta*, 1924, vii, 1039.
4. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, 1923, lvi, 149.
5. Felton, L. D., *Boston Med. and Surg. J.*, 1924, cxc, 819.
6. Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 263.
7. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xx, 429.
8. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1925, xlii, 367.

RHEUMATIC FEVER.*

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Three diseases causing very great economic loss are tuberculosis, syphilis, and rheumatic fever. Not only are the early acute stages of these maladies time-consuming in a period of life when the victims are of greatest value to the community, but their late manifestations, a consequence of their inherent chronicity, cause many individuals to be so severely crippled that they are often less efficient productive units.

About the first two, many positive facts are known; they have clinical, histo-pathological, and immunological points of similarity. Even though their respective causative agents occupy different positions in the biological scale, some of their manifestations are similar allergic expressions. In both we know the etiological agent: In one of them, syphilis, even though much had been learned empirically concerning the effect of mercury and iodids in an era of purely clinical study, the discovery of the Treponema pallidum led to important development in specific therapeutics. In the other, although the tubercle bacillus has been known for many years, no striking specific therapeutic measures have been derived from this knowledge. The information at our disposal, on the other hand, has led to important measures in the prevention of the disease; also, to a knowledge of the natural history of the tuberculous infection. The etiology of rheumatic fever is still undecided, hence we are forced to compare it with infections the nature of which are better understood.

We shall, therefore, draw attention to several points of similarity between these three diseases. I am assuming that rheumatic fever is an infection, with a great variety of manifestations; some short in duration and apparently inflicting but little damage on the tissues of the patient; others having a chronic persistent course from the

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onset with marked evidence of deep-seated injury to the affected organs; and still others in which there are alternate periods of activity and latency.

In making these comparisons it is possible to imagine ourselves in a period where the etiological agent in all three diseases was unknown. At that time many conditions were confused with tuberculosis: phthisis, consumption, often included diseases not due to the tubercle bacillus, although in the majority of instances this was the offending microörganism. Even now, the differential diagnosis in certain patients may rest, not upon the determination of the presence of the tubercle, but rather upon discovery of some other causative factor.

In such an attitude must we often approach our patients with rheumatic fever. It is not possible to be absolutely dogmatic about many cases. Some have symptoms that help in classifying them quickly; about others we are less certain. Doubtless numerous conditions other than rheumatic fever have been and still are included under the term rheumatism. The recognition of many forms of bacterial arthritis has helped to narrow the field so that there is less confusion in differentiating the various diseases with inflammation of the joints as their chief features. The characteristic response of some manifestations of rheumatic fever to certain drugs, such as salicylates, is also a diagnostic aid, just as iodids were formerly used in the differential diagnosis of syphilis. These differential diagnostic methods, however, merely help in bringing certain conditions into a narrower nosological zone; they do not offer much assistance in pointing to the true nature of the disease.

For this purpose we must not only study the manifestations of rheumatic fever directly, but must compare them with other conditions of an apparently similar nature and apply the positive information we have concerning the latter to the malady less well understood. But in making these comparisons it is well to keep constantly in mind that the diseases under consideration are not parallel in all respects. It is in the point of divergence that the peculiar picture of disease is brought out so that each infection has more or less specific characteristics by which it is recognized as a separate entity. Thus the clinical manifestations of an infection are often determined because

of the organs or tissues in which the virus is localized and the specific pathological responses occur. For example, in tuberculosis the lung seems to be most often involved because of its peculiar anatomical structure, and also because of a position favoring implantation of the tubercle bacillus. Similarly, in rheumatic fever the connective tissues in close apposition to large endothelial-lined body cavities or to synovial membranes seem to be especially predisposed to involvement. Nevertheless, if phthisis or scrofula were the only tuberculous manifestations recognized by us we would miss many important features of tuberculosis, so in the consideration of only arthritis and endocarditis we obtain an incomplete picture of rheumatic fever.

In the discussion concerning the relation of heredity and environment to the etiology of tuberculosis, and in the consideration of its contagious nature, the long periods of latency were very confusing factors; it is now well understood that five or ten years may elapse between the time of obvious opportunity of acquiring the infection and the appearance of symptoms. As Opie[9] remarks: "Tuberculosis has so few features of a contagious disease that its infectious nature was in dispute until Villemin's successful experimental inoculations and Koch's demonstration of the tubercle bacillus." It seems probable from clinical observation that similar periods of latency exist in cases of rheumatic fever and that comparable factors in heredity and environment play a role in the etiology of both diseases. For example, Cheedle[1] states that 70 per cent of his patients with rheumatic fever in private practice showed more than one case in the same family. Lately, St. Lawrence,[12] in a study of 100 families with rheumatic fever and 100 with tuberculosis in the St. Luke's Hospital Outpatient Department, showed that practically one-half the families in each group had 2 or more cases per family. There are also several important studies pointing to the possibility that house infections occur in rheumatic fever. It is, therefore, especially difficult to evaluate properly the relation of heredity, direct family infection, and house infection in the etiology of this disease. A recent report of Grenet[5] upon five distinct epidemics of rheumatic fever among Italian troops suggests strongly that direct infection from one person to another may take place and strengthens the opinion that the disease is due to a specific etiological agent.

In studying the various types of manifestations in patients of different ages we are struck by the fact that in both tuberculosis and rheumatic fever severe general acute manifestations are more frequent in the young than in the old. In succeeding decades of life there is a transition from generalized manifestations to more localized lesions. This we know in tuberculosis to point to the development of an immunity or rather an allergy to the infectious agent. Perhaps a better example of this general principle is seen in syphilis where during the early secondary period there is a wide-spread dissemination of the Treponema pallidum and a generalized macular eruption. With each relapse these manifestations tend to become more and more localized and each individual lesion to be more deep-seated and nodular. We now recognize this development of allergy to be an effort on the part of the body to restrict the activity of the virus to smaller and smaller areas. In tuberculosis this allergy is manifested in adults by a greater frequency of chronic tuberculosis of the lung. In rheumatic fever a similar tendency may be seen in the development of more frequent and severe joint lesions in adults than in children, possibly by a tendency to development of chronic arthritis. It does not require a great stretch of the imagination to picture the progressive thickening of the heart valves, the development of the mitral stenosis, the progressive increase of an aortic insufficiency, as sequelæ of the development of an allergy similar to that seen in tuberculosis. Sclerosis, the development of firm connective tissue from an inflammatory base, is a well-recognized feature of later periods of both diseases.

Let us return for a moment to an earlier age period, namely, that of childhood. Involvement of the central nervous system is seen relatively more often in the young than in adults. In children, tuberculosis often manifests itself as a generalized miliary tuberculosis, not infrequently, as a tuberculous meningitis. Similarly, chorea is a common manifestation of generalized rheumatic fever in the young. Accompanying tuberculous meningitis in a child we expect to discover other foci of infection in the lung or perhaps in all the viscera; similarly, in the presence of chorea we are not surprised to find cardiac lesions and frequently wide-spread subcutaneous fibroid nodules. In tuberculosis the severe manifestations usually lead to death; in rheumatic fever, the type of infection, being less severe, leads to a

chronic disease which most often expresses itself as a persistent carditis.

A variation in the susceptibility of various systems of the body at different ages has already been suggested. These systems need not necessarily all be simultaneously involved. In other words, if the virus be generally disseminated, all of the organs do not necessarily respond to this invasion at the same time. For example, although we usually see in children extensive evidence of involvement of the various structures, the child at one time may have chorea, a little later subcutaneous nodules, and still later evidence of marked cardiac disease. The order of involvement does not necessarily follow that given above. In fact, we have examples of nodules with cardiac symptoms, or nodules alone and later chorea. Only one type of manifestation may be detected for a long time; and this fact supports the conception that chorea or subcutaneous nodules occurring in the absence of any other symptoms are really rheumatic in nature.

The tendency for a person to have several attacks of rheumatic fever has long been known. Two points of view regarding this phenomenon are possible; the first is that he is susceptible to the peculiar type of infection and that each attack is the result of re-infection in the same manner as we regard several attacks of lobar pneumonia in one individual as due to different types of pneumococci. The second conception is that the various acute attacks are evidences of relapses of an infection having alternate periods of latency and activity. This view predicates that the patient is infected but once and that clinical recovery is due to the development of a partial immunity resulting in an incomplete elimination of the infectious agent from the body; later some depressing influence lowers the patient's resistance and the dormant virus again becomes active. The well-known chronicity of rheumatic fever in children is good evidence of the capacity of the infectious agent to persist in the body; and it is not rare to encounter similar examples in adults.

All grades of transition between these very chronic cases and those showing rapid and apparently complete recovery without the intervention of drugs are seen. Not infrequently one meets patients with the signs of chronic infection in evidence for many months, in whom finally the inflammatory process seems to have come to an end·

in other words, the patients even with marked cardiac disease have apparently overcome the infection. I use the word "apparently" because experience has taught us to assume the same attitude toward recovery as those responsible for the care of tuberculous patients take in using the term "arrested." In both instances the physician is prepared to see at any time a reawakening of activity.

Unfortunately the etiological agent of rheumatic fever has not been demonstrated conclusively; nor has it been possible to reproduce in animals the characteristic clinical or histo-pathological picture of this disease. This may be due to the absence of the causative agent in the material injected or to the failure of the lower animals to react in the same manner as man to the same irritating substance. Many consider the disease to be due to the so-called Streptococcus rheumaticus, which is really the nonhemolytic streptococcus; but I do not think this opinion can be unqualifiedly accepted. The endocardial vegetations and myocardial lesions so characteristic of the disease known as subacute bacterial endocarditis are also seen in animals properly inoculated with streptococcus viridans. The characteristic microscopic lesions of rheumatic fever, namely, Aschoff bodies are not found in the hearts of either these patients or animals. How then are we to regard the occasional recovery of nonhemolytic streptococci from the blood or gross lesions of patients with rheumatic fever? Up to the present we[6] have been unable to place the strains so recovered in any small number of biochemical or immunological classes such as has been done with the pneumococcus, streptococcus scarlatinæ, or streptococcus erysipelatis. Furthermore, we have been unable to demonstrate in the serum of any rheumatic fever patient a specific agglutinin or precipitin against the homologous streptococcus previously recovered from his blood or lesions. We[15] have been able to demonstrate complement binding antibodies against the nucleoproteins of streptococci, both green and hemolytic, but could find similar antibodies in the serum of convalescent pneumonia patients and in apparently normal individuals. R. C. Lancefield,[7] in our laboratory, has shown that this cross fixation is probably due to the immunological similarity of nucleoproteins of hemolytic streptococci, nonhemolytic streptococci, pneumococci, and, to a smaller extent, of staphylococci. It is, therefore, impossible to use positive results

obtained in complement-fixation tests with antigens containing nucleoproteins of streptococci as conclusive proof that they are the etiological agents; in other words, it is difficult, if not impossible, to use the complement-fixation reaction to detect specific infections with hemolytic and green streptococci. Nevertheless, recent interesting and important studies concerning the nature of streptococcus infections indicate how necessary it is to maintain an open mind as to the relationship between these microörganisms and rheumatic fever.

In the absence of a specific etiological agent or of an immune reaction to give us an indication of uniformity of this disease we are forced to search elsewhere for this clue. Histo-pathology in this respect has furnished us with valuable information in more than one disease. For example, in syphilis the perivascular granuloma is the general type of response to the irritating action of the Treponema pallidum. Straus[13] has defined tuberculosis as a general disease characterized by miliary eruptions in various organs. One might paraphrase this definition in describing rheumatic fever as a general infection characterized by submiliary nodules in certain organs. In the rheumatic heart as already mentioned this nodule has its most characteristic appearance as the Aschoff body; it may be interpreted as a focal interstitial inflammation, situated in the tissue around the smaller arteries and arterioles; there is both a stimulation of fixed cells somewhat similar to the endothelioid cells of the tubercle and an invasion of wandering cells. The peculiarity of the lesion is the presence of "irritation cells," giant cells of a different type than those seen in the tubercle. There is practically always necrosis of the center of the nodules as well as of the contiguous muscle fibers. The healing of the lesion is attended with scar formation.

While the picture of the Aschoff body is not completely reproduced in the other focal lesions of rheumatic fever, the differences can be largely explained upon differences in the tissue involved and the presence of a superimposed exudative process. In the mural endocardium nodules identical in appearance have been found; their presence in great numbers in the left auricle has recently been shown by MacCallum[8] to give a peculiar gross appearance to the lining structures. It seems to us that essentially the same process is respon-

sible for the valvular changes. In most descriptions of rheumatic endocarditis the chief stress is laid upon the very obvious verrucæ; but careful microscopic examination of the entire valve practically never fails to reveal important interstitial changes occurring throughout the major portion of the leaflets. The existence of blood vessels and of areas of inflammation in long-standing rheumatic valvulitis is well recognized and has been explained as secondary to the injury of the endocardium and formation of verrucæ. This conception predicates that the initial injury of the valve is due to the deposition of the causative agent in the cells of the valvular endothelium, then a death of the contiguous structures, followed by a deposition of the various thrombotic elements from the blood stream to form the verrucæ. Recently we have had the opportunity of examining the tissue from 4 patients dying of this disease within two or two and a half weeks of the appearance of the arthritis; in 3 the attack was the first one noted by the patient; in the fourth it was the second. In the first no verrucæ were detectable macroscopically, but many typical submiliary nodules were found in the valves and adjacent chordæ; numerous areas of perivascular inflammation were present throughout a thickened valve; and occasionally definite endarteritis was discernible in the smaller vessels. The entire valve was swollen; but only in one small area of the endocardium was there evidence of necrosis and early verruca formation. The second case showed similar vascular changes and areas of focal inflammation throughout the valvular tissue, a few typical submiliary nodules and slightly larger flat verruca formation in one small area. In the third patient the fatal issue occurred during tonsillectomy on the nineteenth day when it was felt that he had entirely recovered from the acute disease. A small verruca was found at only one place on the aortic valve, but in the substance of the valve there was distinct evidence of mild interstitial inflammation. The death of the fourth patient occurred on the fourteenth day of the second attack of the disease. Grossly no vegetations were seen on any of the valves, but microscopically there were present many foci of intense infiltration with polymorphonuclears, lymphocytes, and a few large endothelioid cells. In a few small areas there was desquamation of the valvular endothelium and very slight deposit of fibrin which was interpreted as early evidence of verruca formation.

From the clinical study of rheumatic polyarthritis we know that swelling due to the exudation of edema fluid and wandering cells can be a marked feature of the inflammatory response. In the valves above described there was also evidence of a similar swelling. It seems to us, therefore, that the primary reaction in rheumatic disease of the valves is in the interstitial tissue, and that the verrucæ are usually due to the deposition of thrombi on a portion of the valve where the vitality of the endothelial and subendothelial layers of the endocardium has been impaired as a result of repeated impacts with the contiguous valve. This theory of death of the endocardium being due to mechanical trauma of an already inflamed structure offers a rational explanation of the localization of the vegetations at a distance from the free margin of the valve. It is also possible that single small verrucæ might form at a point where a submiliary nodule broke through the endocardium. Cary Coombs[3] has marshalled convincing evidence that interstitial valvulitis is the important feature of rheumatic endocarditis.

The recent extensive investigation of Pappenheimer and Von Glahn[10] indicates that essentially the same type of response is ordinarily present in the aorta of patients dying as a result of rheumatic carditis. Upon first glance it might seem that pericarditis was an entirely different process; but in inflammations of this structure the outpouring of serum and fibrin is the usual response to injury whether this be due to the tubercle bacillus or to the causative agent of rheumatic fever. The thick layers of fibrin and subsequent organization may mask the primary lesion; but we have found in the pericardium bodies closely resembling Aschoff bodies, and in the fibrous tissues of adherent pericardiums active perivascular focal reactions closely resembling those found in sclerotic valves.

In the subcutaneous fibroid nodules we have lesions grossly more comparable with tubercles. When excised early they have a similar yellowish gelatinous appearance. Microscopically they are seen to consist of central areas of necrosis containing a small amount of fibrin; surrounding this there are perivascular collections of many large polygonal and branching cells with basophilic protoplasm, lymphocytes, and polymorphonuclear leukocytes in varying numbers. In subcutaneous nodules removed soon after their appearance I[*]

have seen polynuclear cells closely resembling the "irritation giant cells" of the Aschoff bodies. Large subcutaneous nodules may be considered as conglomerations of many small submiliary nodules. Poynton and Holmes[11] describe perivascular submiliary nodules in the brains of patients dying from chorea. The most rational explanation of the symptoms of chorea is the presence of focal areas of encephalitis. At times the meninges may be involved; but encephalitis may occur without meningitis just as myocarditis may occur without pericarditis.

The response of the tissues about the joints to the virus of rheumatic fever is now known to be essentially the same as that found elsewhere. Both Coombs[2] and Fahr[4] have described focal proliferative inflammation in periarticular tissues obtained postmortem from subjects dying from rheumatic heart disease. Recently we[14] have excised bits of joint tissue from patients during the first week of an acute attack and found vascular and perivascular lesions in every respect similar to those present in subcutaneous nodules. When the patient had received no antirheumatic drugs there was, in addition, marked edema of the tissues; when he was fully under the influence of salicylates the exudative features were less prominent, but submiliary proliferative lesions were still easily found.

Combined clinical and histo-pathological studies have, therefore, taught us that there are two general types of response on the part of the body to the invasion of the causative agent of this disease, namely, proliferative and exudative. At the bedside the only visible proliferative lesion is the subcutaneous nodule; but the interpretation of many symptoms and signs as well as of the peculiar course of the disease is made simpler by the knowledge that comparable changes are occurring in important organs. The exudative response, on the other hand, is more evident in the swelling, pain, and tenderness seen in acute rheumatic polyarthritis. Probably the most characteristic feature of this disease is the disappearance of exudation and the symptoms dependent upon it following the exhibition of sufficient doses of certain drugs. It is well established, nevertheless, that subcutaneous nodules may appear continuously in a patient who is receiving maximum doses of salicylates. The failure of the salicylates to influence markedly the proliferative lesion probably explains why the symptoms of chorea persist and the development of valvular

disease continues in patients who are receiving apparently full thera-
peutic doses of salicylates or neocinchophen.

It may be well to mention certain other peculiarities attending the
two types of response; at the same time keeping in mind that usually
there are combinations of the two, and that the clinical picture is
dependent upon which one predominates. With acute toxic symp-
toms and high fever one expects to encounter exudative manifesta-
tions; as the course becomes chronic the fever is lower and symptoms
depending upon the existence of proliferative tissue alterations in the
involved organs become increasingly predominant. There are, more-
over, cases in which a low grade, chronic course is always the outstand-
ing feature. Doubtless many patients with this type of infection
never come under the care of the physician until they have marked
cardiac disease. It has been brought to our attention that in many
parts of the world there seems to be almost a complete absence of
acute articular rheumatism and still mitral stenosis is not infrequent.
If we regard this form of valvular disease as practically always of
rheumatic origin we may assume that the disease as it exists in those
localities assumes a chronic proliferative course from the beginning,
either due to the fact that the etiological agent in those regions is more
attenuated in character or that the persons affected have an inherently
different type of immunity.

It is possible that a changing character of the infection explains the
diminution in the frequency of the condition known as acute inflam-
matory rheumatism; but in this respect it must be borne in mind
that the use of salicylates is one of the most common of practices.
Indeed, it is almost necessary to write a new clinical description of the
disease with the patient under the influence of partial or complete
therapeutic doses of the various antirheumatic drugs. A study of the
older literature makes it evident that in most instances the infection
is self limiting: at times after two weeks, at other times after two, or
three months, and in many instances after longer periods. If a
patient is kept under the influence of salicylates the obvious clinical
manifestations of recurring or continuing infection are often masked.
It is necessary, therefore, to document more carefully the slight symp-
toms that persist, and to resort to more careful instrumental examina-
tion of the patient to detect signs that would otherwise escape our

notice.   Thus we have found that curves of leukocyte counts made at weekly intervals in most cases serve as a fair index of the persistence of infection.   It is true that in some patients there were other evidences of active disease even in the presence of repeated normal leukocyte counts; and rarely in others abnormally high counts have persisted in spite of the absence of other evidences of persisting infection.   As clinicians, we should recognize the necessity of properly evaluating symptoms and signs and should not disregard certain ones because in some exceptional instances they have not followed the accepted rule.

Comparison of Electrocardiographic Evidence of Myocardial Disturbance with Auscultatory Evidence of Valvular Disease and Pericarditis in 81 Cases of Rheumatic Fever.

| | Total cases. | Increase in P–R time. | | | Incomplete heart block. | Change in form of electrocardiogram. | Valvular disease. | | | | Pericarditis. |
		Number.	With fever.	Without fever.			None.	Doubtful.	Mitral.	Aortic.	
First attack..................	34	29	22	21	4	33	5	12	16	5	6
Recurring attacks.............	34	30	24	13	2	29	1	5	28	8 (8?)	3
Cardiac type..................	13	11	10	4	4	13	..	..	13	7 (4?)	7
Total.....................	81	70	56	38	10	˙75	6	17	57	24	16
Per cent....................	..	*87*	*69*	*47*	*12*	*93*	*7*	*21*	*70*	*30*	*20*
								28			

Practically all statistics of the relative frequency of involvement of the heart in rheumatic fever rest upon the occurrence of murmurs which the examiner interpreted as due to deformities of the valves. Pericarditis, obviously, is an indication of very severe cardiac injury. Study of the heart's action with the electrocardiograph has furnished us with evidence that there is some degree of functional disturbance in over 90 per cent of patients suffering from this disease (see Table). While it is impossible to be absolutely certain that these electro-cardiographic alterations are caused by structural changes in the heart, and it has been shown that the administration of digitalis, ether, and

histamine may produce similar alterations in the electrocardiogram, our knowledge of the histo-pathology of rheumatic myocarditis permits us to assume with a fair degree of assurance that these abnormal electrocardiograms are the result of some injury to heart muscle or conduction system. It should be pointed out that these unusual curves in many instances have been only transitory and that upon recovery from the acute infection the form of the curve and the function of the heart have returned to normal and remained in that condition for many years. It may be that changes in conduction time or in form of the ventricular complex are caused by a transitory edema about a few submiliary foci which of themselves are relatively unimportant. A very valuable conclusion to be drawn from this type of evidence, however, is that the heart is probably affected to some degree in practically every patient suffering from rheumatic fever. This conception should eventually alter our attitude toward the therapeutics of this disease.

Other indications of myocardial implication during the period of acute infection are transitory precordial pain and hyperesthesia, and the occurrence of abnormal rhythms; among these the most common is gallop, apparently caused by a market exaggeration of the normal third heart sound. We have noted repeatedly that such gallops were accompanied by distinct alterations in the form of the electrocardiogram. The interpretation of the murmurs so frequently heard during this disease must often await the results of observations extending over months. The point to be especially emphasized is that a search for murmurs is only one of a number of examinations it is necessary to make in order to detect evidence of cardiac derangement.

It has long been recognized that an impaired nutritional state was a feature of the rheumatic infection. Clinical descriptions made in the presalicyl era indicate that marked emaciation was frequently seen in those patients with the subacute and chronic form of the disease. One distinct benefit from antirheumatic drugs is that with the control of the fever and its concomitant toxic state the tendency for the patient to lose weight is less marked. The weight curve, nevertheless, is still a good auxiliary guide in helping us to determine whether or not the patient is succeeding in overcoming his infection.

Not infrequently one notes a continual loss in weight when other symptoms seem to be fairly well controlled by salicylates; also relapses are often heralded by declining weight. There is a more or less common, but unfounded belief that errors in diet or metabolism are important etiological factors in rheumatic fever. These opinions arose from the lactic acid theory of rheumatism, from confusion with gout, and lately from the hypothesis that arthritis is due to a high blood sugar. As a result of these opinions has arisen the practice of omitting fruit to eliminate certain organic acids, red meat "to combat uric acid," and reducing carbohydrates to lower blood sugar. As a result of this régime many of the patients suffer from undernutrition with a consequent deleterious influence on their natural powers of resistance.

Assisting a patient to overcome his infection by increasing his general nutrition has been shown to be one of the most important therapeutic measures in tuberculosis. Maintaining or increasing a typhoid fever patient's weight has been shown to shorten the period of convalescence from that disease. In our experience the maintenance of nutrition is one of the most important therapeutic measures for rheumatic fever.

Analysis of a large series of charts of rheumatic fever patients, in which have been documented all of the signs and symptoms as well as the various laboratory records of activity of infection, has convinced us that the drugs we have been considering as specifics are probably antisymptomatic in their action; and that if patients are carefully studied while under their influence not infrequently signs of persisting infection will be discovered. By means of salicylates or neocinchophen, distressing symptoms of arthritis are fairly easily controlled and high fever is eliminated with a corresponding reduction of the pulse rate. This doubtless spares the heart in more than one respect. If edema is eliminated from the valves by the drugs in the same manner as from the periarticular tissues it is conceivable that the endocardium might be spared some of the traumatic injury to which we think a swollen valve especially liable. A 20 or 30 per cent reduction of the heart rate reduces the number of impacts of the injured valve by a corresponding amount.

All of these are desirable aims of therapeusis; but if in attaining

them the patient is led to believe that he is cured of his infection, or the physician is blinded to the pathological processes that are persisting in spite of the absence of obvious symptoms, the patient may in the end suffer as much permanent injury to some important organ as if he had passed untreated through an attack of the disease. Probably most physicians can remember many patients who have been allowed to be up and about in the wards or in their homes while under the influence of therapeutic doses of salicylates or neocinchopen, and who had relapses when these drugs were discontinued. A common practice is to discharge from the hospital patients while under the influence of these drugs. The reason for this is obvious. The physician often believes that the elimination of symptoms by salicylates is a sign of eradication of the disease; and in a desire to turn over the service as rapidly as possible the doctor yields to the importuning of an apparently well person to return to his home. We may profitably revert to our comparison of syphilis and rheumatic fever in their response to certain drugs. The disappearance of gummata following the administration of iodids was not a sign of the elimination of the treponema from the patient; nevertheless iodids have an important place in the treatment of a patient with syphilis and often help in saving an important organ from irreparable damage. Other therapeutic measures must, however, be applied if the patient is to be cured. Similarly in the treatment of rheumatic fever the beneficial effects of salicylates must be complemented by other measures which have been shown to be of benefit in combating infection.

At this point it may be asked what have been the influences of our drug therapy upon the development of chronic cardiac disease, as well as upon the death rate from rheumatic fever itself. Both the impressions of physicians and vital statistics seem to indicate that the death rate from rheumatic fever is less than in former years (see Chart I). Death during the acute stages of rheumatic fever is usually due to one of three causes: Hyperpyrexia, severe general intoxication, and acute heart failure resulting from severe myocarditis. Doubtless the use of the drugs at our disposal has been instrumental in eliminating to a large extent the first two of these lethal complications. The lower death rate may be due, therefore, in part to a decreased incidence of rheumatic fever as a result of the application of

general hygienic measures and also to better treatment during the acute stages with a consequent result that the condition is one that is recorded as cardiac disease instead of rheumatic fever.

The failure of health departments to make rheumatic fever reportable leaves us without any source of information as to whether or not there is a decreased incidence of this disease. Likewise our idea as to trends in the incidence of heart disease must be based upon mortality rates. In the Registration Area of the United States, vital statistics show that deaths from cardiac disease of the entire population are steadily increasing; but these trends can be explained to a certain degree by the fact that as the expectation of life increases
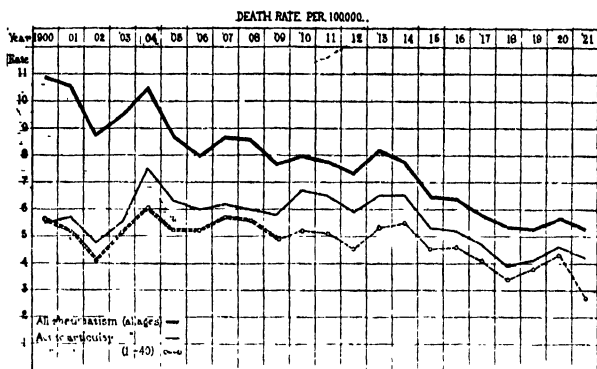
CHART I. Death rate from all rheumatism, and from acute rheumatic fever in New York State, 1900–1921.

there is more opportunity for the degenerative cardiovascular conditions to be an increasingly important factor in the death rate. If we are to obtain an approximate idea of the trends in rheumatic heart disease we must select age periods when we think the rheumatic infection is the most active. Analysis of the deaths from heart disease, endocarditis, and pericarditis in New York State from the years 1900 to 1921 show practically no diminution in the rate of this period (see Chart II). Certain distinct cycles are noted with more deaths in the years of influenza epidemics followed by a decrease in the two or three subsequent years, and then a slowly rising rate until the next epidemic. Life insurance data indicate an increasing rate since 1921. Comparison of the death rates from heart disease

and pulmonary tuberculosis in New York City in the past six years, shows that in children of school age, heart disease is now three times more frequently a cause of death than pulmonary tuberculosis, and that in later age groups with the fall in tuberculosis death rates, there is a slight but steady increase in deaths from heart disease. Insofar as vital statistics are of value in judging the effect of our therapeutics, it seems that both the preventative and curative measures so far applied to infectious heart disease have been of little value.

DEATH RATE PER 100000 (AGES 1-40 INCLUSIVE)

Year |900 '01 '02 '03 '04 '05 '06 '07 '08 '09 '10 '11 '12 '13 |14 15 16 '17 '18 '19 '20 '21

Heart disease ▨ Endocarditis ▥ Pericarditis ☐
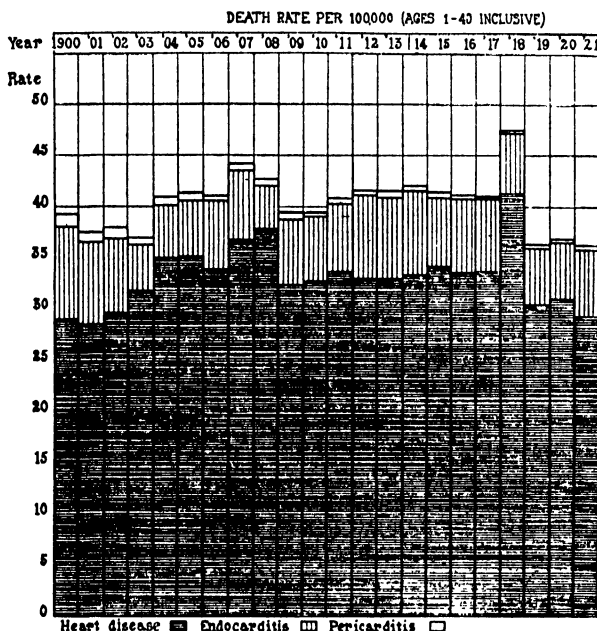
CHART II. Specific death rates (ages one to forty years inclusive) for heart disease, endocarditis, and pericarditis, in New York State, 1900–1921.

We may well inquire the reasons for this failure, and ask whether conditions may be remedied.

Successful prevention of an infection depends primarily upon a knowledge of the nature of the etiological agent, how it enters the body, where it increases in the body, and whence it emerges from the body. Attention has already been drawn to our ignorance of most of these factors in regard to rheumatic fever. Protective immunization depends upon our ability to manipulate the etiological agent

successfully. Chemotherapeutics as applied to prevention of a disease must be founded upon positive demonstration that the drug applied is effective in reaching and destroying the etiological agent and not simply in alleviating symptoms. The successful warfare against tuberculosis has been based not only upon the destruction of the tubercle bacillus in the material in which it was excreted from the body, but also upon a recognition of the chronic nature of the infection and the application of suitable measures to enable the patient to combat that type of infection.
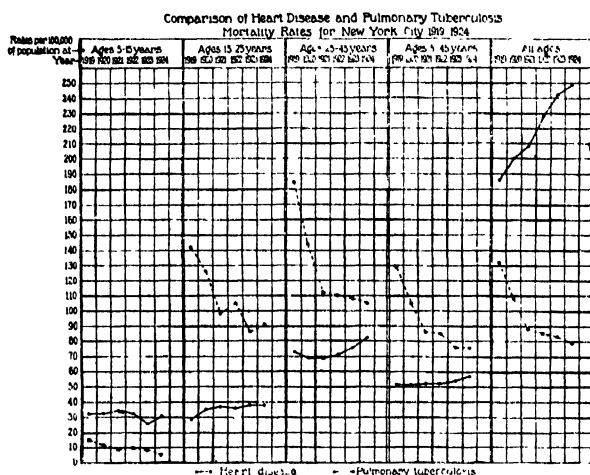
CHART III. Comparison of death rates from heart disease and pulmonary tuberculosis for certain age groups in New York City, 1919–1924.

This brings us directly to what I regard as probably the most important phase of treatment of rheumatic fever, namely, the length of time it is necessary to keep the patient quiet. Briefly, it can be stated that this should be as long as signs of infection persist. Recognition of the different types of the disease already mentioned and the determination into which of these types a patient falls is most useful; for it is obviously unwise to keep a patient with a short course of rheumatic fever in bed a long period, and conversely permitting a patient with a drug masked active infection to be up and about is not the best measure to conserve his strength, protect his heart, and increase his powers of resistance.

The period of rest needed is longer than is usually recognized or enforced. A summary of the length of time it was necessary to keep 72 of our patients in the Rockefeller Hospital is shown in Chart IV.

This period was only that spent in hospital and does not include an average of from two to six weeks in a convalescent home after leaving the hospital.

Let us make clear that these patients were treated according to the accepted methods, that is, antirheumatic drugs for arthritis and high fever, removal of such diseased foci as tonsils and teeth, and
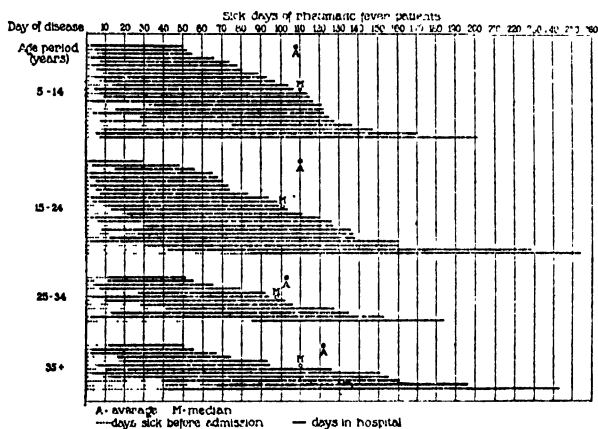
CHART IV. Number of days spent in hospital by 72 patients with rheumatic fever, before they were able to go to a convalescent home. Each line represents time of 1 patient.

treatment of sinuses or other possible infected foci. Our special care was to keep patients quiet until signs of active infection had passed.

If general hospitals kept their patients with rheumatic fever for periods similar to this there would be none other than rheumatic fever patients in the wards during the winter and spring months. The question to be met is: "How can these patients be given proper periods of rest?" In this one point of proper provision for rest cure the institutional and sanatorium treatment of tuberculosis has pointed a way. Perhaps the time may come when the tuberculosis problem will be completely solved and these sanatoria will be converted

into hospitals for the treatment of patients with rheumatic hearts; but before that time it is to be hoped that provision for the proper care of a large group of these patients will have been made. A few heart hospitals where proper study of these problems could be both intensively and extensively carried out would do much toward formulating a definite plan in the treatment of a condition which up to the present has been but little permanently benefited by the methods now in vogue.

BIBLIOGRAPHY.

1. Cheedle, W. B.: Allbutt and Rolleston, System of Medicine, 1908, 2, Part I 647.
2. Coombs, Cary: Jour. Path. and Bacteriol., 1911, 15, 489.
3. Coombs, Cary: Rheumatic Heart Disease, Bristol, 1924.
4. Fahr, T.: Virchow's Arch. f. path. Anat., 1921, 232, 134.
5. Grenet, H.: Gaz. d. hôp., 1920, 93, 5.
6. Kinsella, R. A., and Swift, H. F.: Arch. Int. Med., 1917, 19, 381.
7. Lancefield, R. C.: Jour. Exp. Med., 1925, in press.
8. MacCallum, W. G.: Bull. Johns Hopkins Hosp., 1924, 35, 239.
9. Opie, E. L.: Am. Rev. Tuberculosis, 1922, 6, 525.
10. Pappenheimer, A. M., and Von Glahn, W. C.: Jour. Med. Res., 1924, 44, 489.
11. Poynton, F. J., and Holmes, G.: Lancet, October 13, 1906, p. 982.
12. St. Lawrence, W.: Jour. Am. Med. Assn., 1922, 79, 2051.
13. Straus, quoted by Cornet: Nothnagel's Encyclopedia of Practical Medicine, American edition, Philadelphia, 1907.
14. Swift, H. F.: Jour. Exp. Med., 1924, 39, 497.
15. Swift, H. F., and Lancefield, R. C.: In press.
16. Swift, H. F., Miller, C. P., Jr., and Boots, R. H.: Jour. Clin. Investigation, 1924, 1, 197.
Statistics for New York State based on the Reports of the Bureau of the Census; for New York City, on the Bulletins of the Department of Health, New York City.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

VIII. THE DISTRIBUTION OF HYDROGEN, CHLORIDE, AND BICARBONATE IONS IN OXYGENATED AND REDUCED BLOOD.

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The purpose of this paper is to present data showing the effects of changes in blood reaction and oxygenation on the distribution ratios of hydrion activities and bicarbonate and chloride concentrations between serum and cells, and to discuss the relative activity coefficients of the Cl and HCO_3 anions in serum and cells.

In the fifth paper of this series (1) the laws governing the electrolyte and water distribution between cells and serum in the blood were formulated, and subjected to experimental verification. It was shown that as a first approximation

$$r = \frac{[H^{\cdot}]_s}{[H^{\cdot}]_c} = \frac{[Cl]_c}{[Cl]_s} = \frac{[HCO_3]_c}{[HCO_3]_s} = 1 - f[BHb]_c$$

where $[BHb]_c$ is the amount of base bound by hemoglobin in the cells. The value of $f[BHb]_c$ was expressed in their Equations 10 and 14 of that paper. In the development of these equations it was pointed out that the assumption of complete dissociation of the electrolytes served only for a first approximation. However, if the dissociation (or activity) of the electrolytes in cells and serum were nearly equal it could be expected that the equation would hold approximately. This was found to be the case for the distribution of Cl and HCO_3 in oxygenated blood under varying CO_2 tensions, and for the distribution of $H^{\cdot}$ as calculated from the CO_2 contents by Hasselbalch's equation, $pH = pK_1' + \log \frac{[BHCO_3]}{[H_2CO_3]}$.

In the present paper we have determined the distributions not only in oxygenated but also in reduced blood, in order to ascertain whether the predicted effect of reduction (causing r to move nearer to 1 as a result of the lesser amount of base bound by the hemoglobin) occurs. As will be shown in the experimental part, it does occur, and approximates the magnitude calculated from the effect of oxygenation and reduction on the base bound by the hemoglobin.

We have also determined the H˙ activities in reduced cells and serum directly by the electrometric method, and determined the pK' of Hasselbalch's equation for cell contents. By using this pK' in calculating the H˙ activities by Hasselbalch's equation in oxygenated blood, where the hydrogen electrode cannot be used, the activities thus estimated could be placed on a basis of measured electrometric H˙ activity values. From the relationship between the distribution ratios of H˙ activities and Cl and HCO₃ concentrations between serum and cells we have attempted to estimate the relative activity coefficients for Cl' and HCO₃' in serum and cells. The importance of such estimates was recognized by Warburg (2). We have attempted also to determine the Cl' activities directly by the electrometric method. The difficulties of doing so in the presence of the blood proteins, which Warburg (2) has discussed, have thus far prevented success at this point.

For the determination of chlorides in the cells Van Slyke, Wu, and McLean (1) did not possess a method of satisfactory accuracy. They therefore estimated the initial cell Cl content by difference from whole blood and serum analyses, and calculated the subsequent cell chloride variations, caused by varying CO₂ tensions, from the variations in serum Cl, for which a highly accurate method was available. Accordingly, as they pointed out, their chloride analyses afforded accurate data for the *variation* of the Cl ratio with changing pH, but could be accepted only as approximate for the absolute values of the [Cl]ₑ: [Cl]ₛ ratio. The accurate nitric acid ashing method for cell chlorides (3) developed in the meantime has been used in the present experiments, and we believe the absolute values of the chloride ratios, as well as the shifts in them, given in this paper are fairly reliable. They indicate in agreement with Warburg (2) a [Cl]ₑ: [Cl]ₛ ratio curve definitely lower than the [HCO₃]ₑ: [HCO₃]ₛ curve.

Symbols and Equations.

The symbols used in the present paper are the following.

r = Donnan distribution ratio of diffusible ions between serum and cells.

γ = activity coefficient of ions.

α = activity of ions.

C = molal concentration.

V = valence.

μ = ionic strength.

$[Cl]$ = Cl molality in millimols of chloride per kilo of water.

$[HCO_3]$ = HCO$_3$ molality in millimols of bicarbonate per kilo of water.

s = as a subscript refers to serum.

c = as "   "   "   " cells.

$(B)_s$ = milli-equivalents of serum base per kilo of whole blood.

$(B)_c$ =   "   " cell   " " " " "   "

$(BP)_s$ =   "   " base bound to serum protein per kilo of whole blood.

$(BP)_c$ =   "   " " " " "   "   " " "   "

(Hb) = millimols of hemoglobin per kilo of whole blood.

$(H_2O)_s$ = kilos of serum water   "   " "   "   "

$(H_2O)_c$ = " " cell   "   " " "   "   "

P_{CO_2} = tension of CO$_2$ in millimeters of mercury.

pH^* = $\log \alpha_H\cdot$.

pX = $-\log X$ in general.

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* Sörensen and Linderström-Lang (4) have recently reviewed the various standards upon which pH measurements have been based and have made the following recommendations regarding nomenclature and consistency in calculation. When the 0.1 N calomel electrode and the Bjerrum extrapolation is used the value of the calomel electrode at 18° is taken to be 0.3380 volts, if hydrogen ion values are to be consistent with the hydrogen ion concentration of hydrochloric acid solutions as determined from conductivity measurements. This hydrogen ion value in the logarithm form is to be designated by pH as in the past. If on the other hand, hydrogen ion values are to be consistent with activity values for hydrochloric acid, then the value of the calomel electrode is taken to be 0.3357 at 18°. When this is done Sörensen and Linderström-Lang recommend that the logarithm of the hydrogen ion value be designated by $p\alpha_H$. In their proposed nomenclature $p\alpha_H$ = pH + 0.04.

In the previous papers of this series we have based our pH values on activity values of hydrochloric acid, so that our pH values have been equal to Sörensen and Linderström-Lang $p\alpha_H$ values. In order to avoid the confusion of change in nomenclature in this series of papers, we shall continue to use the symbol pH to express the negative logarithm of H˙ activity.

The following basic equations are used, and will be referred to in the text.

(1)
$$r = 1 - \frac{(BP)_e + (Hb)}{2(B)_e - (BP)_e + (Hb)} + \frac{(BP)_e}{2\{(B)_e - (BP)_e\}} \quad {}^1$$

$$\gamma = \frac{\alpha}{C}$$

$$\mu = \frac{1}{2} \Sigma \, CV^2$$

(2)
$$\log \gamma = -\beta V^2 \sqrt{\mu}$$

(3)
$$\frac{[\alpha_H\cdot] \times [\alpha_{HCO_3}']}{[\alpha_{H_2CO_3}]} = K_1$$

$$pH + p\alpha_{HCO_3}' - p\alpha_{H_2CO_3} = pK_1$$

(4)
$$pH + p[HCO_3] - p[H_2CO_3] = pK_1 - p\gamma_{HCO_3} = pK_1'$$

(5)
$$p\gamma_{HCO_3} = -\log \gamma_{HCO_3} = pK_1 - pK_1'$$

(6)
$$[H_2CO_3] = \frac{P_{CO_2} \times 0.555}{760 \times 0.0224} = 0.0326 \, P_{CO_2}$$

---

[1] Equation 1 is Equation 14 of Van Slyke, Wu, and McLean. In their derivation of it one factor was omitted without the explanation which should be given. The value of $\frac{(H_2O)_e}{(H_2O)_c}$ was shown to equal $\frac{2(B)_e - (BP)_e}{2(B)_e - (BP)_e + (Hb)}$ and the latter was then substituted for $\frac{(H_2O)_e}{(H_2O)_c}$ in their Equation 12. The resulting equation is $r = 1 - \frac{2(B)_e - (BP)_e}{2(B)_e - 2(BP)_e} \times \frac{(BP)_e + (Hb)}{2(B)_e - (BP)_e + (Hb)} + \frac{(BP)_e}{2(B)_e - (BP)_e}$. In their Equation 14 the factor $\frac{2(B)_e - (BP)_e}{2(B)_e - 2(BP)_e}$ is omitted, as though the numerator and denominator exactly cancelled, which is, of course, impossible. However, the value of $(BP)_e$ is only about 0.04 of $2(B)_e$ so that the effect of the factor $\frac{2(B)_e - (BP)_e}{2(B)_e - 2(BP)_e}$ is only to decrease the term subtracted from 1 by about 4 per cent, and to increase the value of r by from 1 to 2 per cent. For the purposes of the paper such a factor could be neglected, and the equation, simplified by omitting it, was given as above.

Ionic Strength of Serum.

Donnan's law expressing the distribution of diffusible ions between two solutions separated by a membrane impermeable to one or more ions was developed from thermodynamic reasoning applied to ideal solutions, in which it is assumed "that the electrolytes are completely ionized and that the ions act like ideal solutes. The exact equations can, however, be stated only in terms of the chemical potentials of Willard Gibbs or of the ion activities or ionic activity coefficients of G. N. Lewis" (5).

Donnan's equations apply therefore to the effective concentrations or activities of the diffusible ions rather than their actual concentrations. (The nature of ionic activity and its relationship to ionic concentration are discussed in Lewis and Randall's "Thermodynamics" (6), particularly in Chapters XXII, XXVI, and XXVIII.) The conception of ionic activity presented by Lewis, and apparently concordant with as many of the known facts as any present conception, is that all strong electrolytes are completely dissociated in solution, but that forces acting between the ions prevent them from exhibiting the same activities that they have at infinite dilution. Lewis speaks of the activity as the fugacity or escaping tendency of a solute as compared with the fugacity in the standard state. (In the case of a solute gas the fugacity is exemplified by its tension.) For solutes in solution the standard state is considered to be that of a solution such that $\frac{\alpha}{m}$ at infinite dilution, where α is the activity and m the molality.

Perhaps a more tangible conception than the above sketched from Lewis and Randall is that the activity of the ions in a given solution may be expressed as the ratio between the amount of work the ions will do on dilution to infinite volume and the amount of work that the same number of molecules of an ideal gas would do in expanding similarly from the same initial volume. The interionic forces opposing removal of the ions from each other diminish the work the latter do on dilution, diminish their fugacity, and evince themselves by their effects on such solution properties as osmotic pressure and electrical conductivity.

Milner from mathematical considerations and Debye and Hückel

from kinetic premises arrived at nearly the same expression for the change in the free energy of a dilute solution of a strong electrolyte when it is infinitely diluted (A. A. Noyes (7)). Taking into account only the effect of the interionic forces, this expression leads to the following relationship between the activity coefficient of any ion (activation, Noyes (7))and the total ionic strength of the solution.

(2) $$\log \gamma = - \beta V^2 \sqrt{\mu} \text{ when } \mu < 0.10$$

Some uncertainty exists regarding the exact value of β. Debye and Hückel's derivation leads to the value 0.50 which coincides with the figure obtained by Brönsted and La Mer (8) from solubility measurements; Milner's derivation leads to a value of about 0.34, and A. A. Noyes thinks that the most probable value is approximately 0.42. As a matter of fact the value actually found experimentally is not exactly identical for all ions, though of the same order of magnitude: $e.g.$ β for Cl is approximately 0.33 (calculated from Lewis and Randall[2]) whereas for HCO₃ it is 0.50 (Hastings and Sendroy (9)).

A more complete expression valid over a wider range of ionic concentrations has been recently given by Hückel (10).

$$\log \gamma = - \frac{\beta \sqrt{\mu}}{1 + A \sqrt{\mu}} + C \mu$$

where A is a function of the ionic diameters and C is a function of the dielectric properties of the solution. Since no information is as yet available concerning the factors A and C in protein solutions it is impossible to utilize the equation at the present time in our system. A first approximation of γ from the ionic strength of the serum and cells has been attempted, however, by the use of the simplified Equation (2) $\log \gamma = - \beta V^2 \sqrt{\mu}$.

To calculate the activity coefficient of strong electrolytes in serum from Equation 2 it is necessary first to obtain an approximate value for the ionic strength of the serum. This has been done for horse serum at pH 7.40 from typical analyses of the constituents and leads to the value $\mu_s = 0.167$. These data, given in Table I, are self-explanatory except in the case of the rôle attributed to the proteins. We have

[2] Lewis and Randall (6), p. 382.

assumed a protein concentration of 80 gm. per kg. of water. The amount of base bound at pH 7.4 is 0.18 millimol per gm. of serum protein (Van Slyke, Wu, and McLean (1)). This means that 0.0144 equivalent of base per kg. of water is bound to protein. It is impossible at the present time to state exactly the effect of proteins on the ionic strength of the serum for the following reasons: The protein molecule is a large one, and the charges due to ionization of the constituent groups are probably so far removed from one another that they cannot be considered to affect the activity of ions in the same manner as other polyvalent ions. It is possible that the surface density of the charges is more significant than the charges per molecule.

TABLE I.

Calculation of Ionic Strength of Normal Horse Serum at pH, 7.40.

Basic constituents.	$\dfrac{\text{Mols}}{\text{Kg. H}_2\text{O}}$	Acidic constituents	$\dfrac{\text{Mols}}{\text{Kg. H}_2\text{O}}$
Total	0.160	Total.	0.160
Na	0.1451	Cl	0.103
K	0.0065	HCO_3	0.029
Ca	0.0032	SO_4	0.002
Mg	0.0010	HPO_4	0.001
		H_2PO_4	0.0002
		Protein.	0.0144
			0.0074

$$\mu_s = \tfrac{1}{2}\,\Sigma CV^2 = 0.167.$$

In the absence of more exact information on this point we have assumed in this calculation that the proteins act as if univalent. Further evidence that the ionic strength of serum is approximately 0.167 is given by the fact that the experimentally determined pK_1' of carbonic acid in serum is 6.13 and that this corresponds to the calculated pK_1' of carbonic acid in a salt solution of ionic strength 0.160.

Using the value $\mu_s = 0.167$ and the equation $-\log \gamma_s = 0.5\, V_2\sqrt{\mu}$ $= 0.5 \sqrt{\mu}$ one may calculate the activity coefficient of HCO_3 ions in serum to be $\gamma_s = 0.625$. As will be seen, this agrees well with the γ value estimated as $\gamma = \dfrac{K_1}{K_1'}$, where K_1 is the dissociation constant of carbonic acid, and $K_1' = \alpha_H \cdot \dfrac{[BHCO_3]}{[H_2CO_3]}$.

Ionic Strength of Cells.

The calculation of the ionic strength of cell fluid presents greater difficulty. In Table II we have presented the data used in its calculation from analysis. As in the case of the serum proteins we have assumed that hemoglobin functions as a univalent component in contributing to the ionic strength of the cell fluid. Calculated in this manner the ionic strength, μ_c = 0.171. The activity coefficient of monovalent ions estimated from the equation $- \log \gamma_c = 0.5 \sqrt{\mu_c}$ is γ_c = 0.62, practically the same as the serum.

TABLE II.

Calculation of the Ionic Strength of Normal Oxygenated Horse Cells at pH_c 7.15.

Basic constituents	$\dfrac{\text{Mols}}{\text{Kg. H}_2\text{O}}$	Acidic constituents.	$\dfrac{\text{Mols}}{\text{Kg. H}_2\text{O}}$
Total $\dfrac{\text{Mols}}{\text{Kg.H}_2\text{O}}$	0.170	Total $\dfrac{\text{Mols}}{\text{Kg.H}_2\text{O}}$	0.170
Na*		Cl	0.800
K	0.170	HCO₃	0.0230
Ca*		SO₄	0.0008
Mg*		Hb	0.0500
		X(HPO₄, H₂PO₄, etc.)	0.0154

$$\mu_c = \tfrac{1}{2} \Sigma C V^2 = 0.171$$

* Bunge reports no Na, Ca, or Mg in horse cells.

Several assumptions have been made in these calculations which render the values of γ_s and γ_c obtained merely first approximations. We have assumed first, that we are dealing with solutions of strong electrolytes; second, that the solutions are not so concentrated that the size of the ions plays a significant rôle; and third, that the dielectric properties of the solutions are not different from those of water. In the case of serum the results make it probable that these assumptions are justifiable. But in the case of the cell fluid, which contains over 30 per cent of protein, the assumptions do not appear to be exact, as will be shown in a subsequent section on the determination of the dissociation constant of carbonic acid in cells.

EXPERIMENTAL METHODS.

Reduced and oxygenated defibrinated horse bloods were equilibrated at 38° with various CO_2 tensions. After centrifugation and transfer to small containers over mercury, determinations of the water, CO_2, Cl, and, where possible, electrometric estimations of the pH, of the serum and cells were made. The technique employed in saturation and separation of the serum and cells was that described by Van Slyke, Wu, and McLean (1). Reduction of the blood was obtained by two saturations with hydrogen.

Carbon dioxide and oxygen analyses of the blood were made in the manometric blood gas apparatus (11). Chloride determinations were made by oxidizing the protein of the serum and cells with concentrated nitric acid in the presence of known amounts of silver nitrate and titrating the excess silver according to the method recently described (3). Electrometric determinations of the hydrogen ion activity were made in the serum and cells of the reduced blood. Electrometric pH values of the oxygenated blood were impossible to obtain because of the effect of the oxygen on the hydrogen electrodes. All concentrations are expressed in millimols per kilo of water.

In all of the electrometric determinations the following procedure has been employed. The cell system used has been composed of

Saturated calomel cell	Saturated KCl bridge	Electrode liquid	H_2 − Pt

A Clark-Cullen electrode vessel bearing a thermometer for the measurement of the temperature of the liquid contained the solution whose reaction was to be measured. All measurements were performed in an air bath at a temperature of 38 ± 0.10°C.

At the beginning of each experiment 0.1 N HCl was placed in the electrode vessel and the resulting potential measured. From this the ϵ of the calomel cell was calculated from the equation

$$\epsilon = \text{E.M.F.} - 1.08 \times 0.06169$$

1.08 is the value for pH of 0.1 N HCl calculated from Lewis and Randall.[2]

When a solution of unknown reaction is in the electrode vessel the pH of such a solution is calculated from the equation

$$pH = \frac{E.M.F. - \epsilon}{0.06169}$$

All potential measurements were corrected to 760 mm. of dry hydrogen (Clark[3]).

The calculation of pH values in this way involves three assumptions:

(a) That the pH of 0.1 N HCl is 1.08 at 38°C.

(b) That whatever diffusion potential may exist between the saturated KCl and the electrode liquid is the same in the case of 0.1 N HCl and the liquid of unknown reaction

(c) That the voltage developed in the hydrogen electrode would be the same in a serum or cell solution which had the same hydrogen ion activity as in the 0.1 N HCl solution with which our system was standardized.

None of the assumptions is certainly exact, but in the absence of more accurate information concerning the activity of ions of strong electrolytes, the magnitude of diffusion potentials, and the effect of serum and, particularly, cell constituents on ϵ we have utilized these assumptions in order to calculate pH values.

Determination of pK_1' of Carbonic Acid in Horse Serum.

The apparent dissociation constant K_1' in Henderson's equation represents the real constant K_1 divided by γ_{HCO_3}. Hence the relationship between K_1' and K_1 may be used to estimate γ_{HCO_3}.

The apparent constant for serum was calculated from experimental data in Table III according to Hasselbalch's equation, $pK_1' = pH - \log \frac{[HCO_3]}{[H_2CO_3]}$, where pK_1' is $- \log K_1'$ and $[HCO_3]$ represents bicarbonate concentration. pK_1' was found to be 6.13. Hastings and Sendroy (9) in a recent paper have determined the value for pK_1 to be 6.33. From these two figures one may estimate

$$\log \gamma_{HCO_3}' = pK_1' - pK_1 = 6.13 - 6.33 = -0.20$$

$$\gamma_{HCO_3} = 0.63$$

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[3] Clark (12), p 459.

Hastings and Sendroy have shown that the relationship between the bicarbonate activity coefficient and the ionic strength of bicarbonate-salt solutions is expressible as

$$- \log \gamma_{HCO_3'} = 0.5 \sqrt{\bar{u}}$$

TABLE III.

Determination of pK_1' of Carbonic Acid and Calculation of $p\gamma_{HCO_3}$ in Horse Serum.

1924	[H₂O]	E.M.F.	Electrometric pH.	Total [CO₂].	CO₂ tension.	[H₂CO₃]	[HCO₃]	log $\frac{[HCO_3]}{[H_2CO_3]}$	pK_1'	$p\gamma_{HCO_3}$
	$\frac{gm.}{cc.}$	$mv.$		$\frac{mM}{kg. H_2O}$	$mm.$	$\frac{mM}{kg. H_2O}$	$\frac{mM}{kg. H_2O}$			
Feb. 23	0.927	706.5	7.61	30.67	30.1	0.982	29.69	1.480	6.13	0.20
	0.937	705.4	7.61	30.90	30.6	0.998	29.90	1.477	6.13	0.20
	0.937	693.9	7.41	32.87	50.2	1.636	31.23	1.281	6.13	0.20
	0.937	694.5	7.43	32.98	49.7	1.620	31.36	1.287	6.14	0.19
Jan. 17	0.933	670.8	7.07	46.64	151.2	4.93	41.71	0.927	6.14	0.19
	0.929	683.4	7.27	38.18	80.6	2.63	35.55	1.130	6.14	0.19
	0.928	692.6	7.42	30.18	43.8	1.43	28.75	1.303	6.12	0.21
Jan. 29	0.918	688.2	7.33	25.10	45.7	1.49	23.61	1.20	6.13	0.20
Mar. 13	0.932	673.3	7.10	47.20	15.50	5.06	42.14	0.921	6.18	0.15
	0.935	696.4	7.47	30.86	42.12	1.37	29.49	1.333	6.14	0.19
Mar. 20	0.934	670.9	7.06	45.22	150.4	4.90	40.32	0.914	6.15	0.18
	0.935	685.1	7.29	33.23	66.0	2.15	31.08	1.160	6.13	0.20
	0.932	699.4	7.52	23.57	26.1	0.85	22.72	1.426	6.09	0.24
Average..........									6.13	0.20

Substituting 0.2 for $-\log \gamma_{HCO_3'}$ one calculates $\mu = 0.160$, which agrees closely with the ionic strength of 0.167 estimated from serum analyses. It therefore appears probable that the three assumptions above stated, involved in the calculation of pH from potential readings, are valid for serum.

Determination of pK_1' of Carbonic Acid in the Cells of Horse Blood.

To determine the pK_1' of carbonic acid in undiluted cell fluid we have hemolyzed cells with saponin, then completely reduced the fluid

by double or triple saturation with hydrogen containing sufficient CO_2 to give the desired pH. From the determined CO_2 tension, CO_2 content, and pH, the pK_1' values were calculated as $pK_1' = pH - \log \frac{[HCO_3]}{[H_2CO_3]}$. From eleven such experiments the pK_1' of carbonic acid in horse cells has been calculated to be 5.93 and $- \log \gamma_{HCO_3} = 0.40$. This would indicate γ_{HCO_3} to be 0.40 as contrasted with 0.62, the value calculated from the ionic strength of cell fluid. This discrepancy we

TABLE IV.

Determination of pK_1' of Carbonic Acid in Reduced Horse Cells.

1924	[H₂O]	E.M.F.	Electrometric pH.	Total [CO₂].	CO₂ tension.	[H₂CO₃]	[HCO₃]	$\log \frac{[HCO_3]}{[H_2CO_3]}$	pK_1'	γ_{HCO_3c}
	$\frac{gm.}{cc.}$	*mv.*		$\frac{mM}{kg.\ H_2O}$	*mm.*	$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg.\ H_2O}$			
Jan. 17	0.661	657.0	6.84	42.90	151.2	4.930	37.97	0.887	5.95	0.38
	0.659	660.7	6.90	33.65	80.6	2.627	31.02	1.072	5.83	0.50
	0.656	673.7	7.12	25.62	43.8	1.430	24.19	1.228	5.89	0.44
Mar. 13	0.692	657.4	6.84	44.08	155.0	5.058	39.02	0.888	5.95	0.38
	0.693	675.7	7.14	26.34	42.1	1.372	24.97	1.260	5.88	0 45
Mar. 20	0.712	660.3	6.88	42.86	150.4	4.90	37.96	0.889	5.99	0.34
	0.699	675.4	7.12	31.46	66.0	2.15	29.31	1.333	5.99	0.34
	0.687	687.9	7.33	19.66	26.1	0.85	18.81	1.345	5.98	0.35
Nov. 30	0.711	664.1	6.97	38.9	112.5	3.67	35.2	0 98	5.99	0.34
	0.711	667.8	7.03	29.2	60.8	1.98	27.2	1.14	5.89	0.44
	0.711	676.3	7.16	19.2	27.4	0.90	18.3	1.31	5.85	0.48
Average...									5.93	0.40

are unable to explain. As stated above, Van Slyke, Wu, and McLean (1) assumed that the pK_1' value in the cell fluid was 6.12, the same as in serum, a conclusion to be expected from the approximate equality of the ionic strength in cells and serum, and from Warburg's (2) finding the same pK_1' in hemolyzed whole blood as in serum. Whether the actual pK_1' in cells is that estimated from the ionic strength, *viz.* 6.12 to 6.13, or that calculated from the electrometric pH measurements, we cannot at present decide. It is possible that the high pro-

tein content of the cell fluid affects the potential readings in such a manner that an error is introduced in interpreting them into pH values by the usual formula. In the absence of further data which would make the calculation of the activity coefficient of the bicarbonate ion in cells more certain we have used the value 5.93 for pK_1' in the calculation of the pH of oxygenated cells.

TABLE V.

Experiment to Determine $\dfrac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c}$, $\dfrac{[Cl]_c}{[Cl]_s}$ *and* $\dfrac{[HCO_3]_c}{[HCO_3]_s}$ *on Reduced Horse Blood.*

Total $[HbO_2]$ = 8.42 mM

No.		[H₂O]	CO₂ tension.	[H₂CO₃]	Total [CO₂].	[HCO₃]	log $\frac{[HCO_3]}{[H_2CO_3]}$	pH	[Cl]	[HbO₂]
		$\frac{gm.}{cc.}$	mm.	$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg\ H_2O}$			$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg.\ H_2O}$
1	Serum.	0.930	151.8	4.955	44.76	39.80	0.905	7.035	101.8	
	Cells.	0.704	151.8	4.955	42.30	37.34	0.899	6.807	79.2	
2	Serum.	0.926	46.2	1.505	30.71	29.20	1.288	7.418	105.8	0.43
	Cells.	0.726	46.2	1.505	24.00	22.49	1.115	7.105	77.1	

	No. 1.	No. 2.
pH$_s$	7.035	7.418
$\dfrac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c}$	0.59	0.49
$\dfrac{[Cl]_c}{[Cl]_s}$	0.78	0.73
$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	0.94	0.77

Determination of the Ratios $\dfrac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c}$, $\dfrac{[HCO_3]_c}{[HCO_3^-]_s}$, $\dfrac{[Cl]_c}{[Cl]_s}$.

In Tables V to IX are given the results of five experiments on the distribution of $\alpha_H\cdot$, Cl, and HCO₃ between serum and cells as affected by changes in pH$_s$ from 7.0 to 7.6. Two experiments (Tables VIII and IX) include data showing the effect of oxygenation and reduction on the distribution of these ions. The pH values given are calculated

from Equation 5 and the pK₁′ values given in Tables III and IV; *viz.*, 6.13 for serum and 5.93 for cells.

<div align="center">TABLE VI.</div>

Experiment to Determine $\dfrac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c}$, $\dfrac{[Cl]_c}{[Cl]_s}$, $\dfrac{[HCO_3]_c}{[HCO_3]_s}$ *for Reduced Horse Blood.*

Total $[HbO_2]$ = 9.75 mM

No.		$[H_2O]$	CO_2 tension.	$[H_2CO_3]$	Total $[CO_2]$.	$[HCO_3]$	$\log \dfrac{[HCO_3]}{[H_2CO_3]}$	pH	$[Cl]$	$[HbO_2]$
		gm./cc.	*mm.*	$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg.\ H_2O}$			$\frac{mM}{kg.\ H_2O}$	$\frac{mM}{kg.\ H_2O}$
1	Serum.	0.935	149.4	4.87	47.13	42.26	0.942	7.072	101.5 }	0.75
	Cells.	0.714	149.4	4.87	43.61	38.74	0.901	6.831	79.4 }	
2	Serum.	0.932	83.9	2.74	39.44	36.70	1.127	7.257	103.7 }	0.67
	Cells.	0.716	83.9	2.74	34.80	32.06	1.068	6.998	76.2 }	
3	Serum.	0.931	47.3	1.53	32.24	30.71	1.311	7.441	106.0 }	0.95
	Cells.	0.703	47.3	1.53	26.70	25.17	1.216	7.146	72.0 }	
4	Serum.	0.929	28.9	0.94	26.36	25.42	1.432	7.562	107.8 }	1.28
	Cells.	0.699	28.9	0.94	21.13	20.19	1.331	7.261	68.8 }	

	No. 1.	No. 2.	No. 3.	No. 4.
pH₈..........................	7.072	7.257	7.441	7.562
$\dfrac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c}$......................	0.57	0.55	0.51	0.50
$\dfrac{[Cl]_c}{[Cl]_s}$......................	0.78	0.77	0.68	0.64
$\dfrac{[HCO_3]_c}{[HCO_3]_s}$..................	0.92	0.87	0.82	0.79

The $[H_2CO_3]$ molality in millimols per kilo of water in both serum and cells is calculated from the equation

$$(6) \qquad [H_2CO_3] = \frac{P_{CO_2} \times 0.555}{760 \times 0.0224} = 0.0326\ P_{CO_2}$$

From determinations of the solubility of CO_2 in acidified serum and in dilute acidified cell solutions it was found that within the limits

of our analytical precision, the other solutes have no effect on the solubility of CO_2 per kilo of blood water. The value of the molality $[H_2CO_3]$ thus calculated is also the value for the activity $\alpha_{H_2CO_3}$.

The $[HCO_3]$ was obtained by subtracting the $[H_2CO_3]$ concentration from the total $[CO_2]$ content.

TABLE VII.

Experiment to Determine $\dfrac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c}, \dfrac{[Cl]_c}{[Cl]_s}, \dfrac{[HCO_3]_c}{[HCO_3]_s}$ *for Reduced Horse Blood.*

Total $[HbO_2]$ = 9.61 mM

No.		[H₂O]	CO₂ tension	[H₂CO₃]	Total [CO₂]	[HCO₃]	log $\frac{[HCO_3]}{[H_2CO_3]}$	pH	[Cl]	[HbO₂]
		gm. cc.	mm.	mM kg. H₂O	mM kg. H₂O	mM kg. H₂O			mM kg. H₂O	mM kg. H₂O
1	Serum.	0.934	151.2	4.930	46.64	41.71	0.928	7.058	101.9	1.09
	Cells.	0.661	151.2	4.930	42.90	37.97	0.887	6.817		
2	Serum.	0.929	80.6	2.627	38.18	35.55	1.132	7.262	103.2	1.30
	Cells.	0.659	80.6	2.627	33.65	31.02	1.072	7.002	73.6	
3	Serum.	0.928	43.8	1.43	30.18	28 75	1.303	7.433	102.4	
	Cells.	0.656	43.8	1.43	25.62	24.19	1.288	7.158		
4	Serum.	0.943	29.8	0.97	26.90	25 93	1.427	7.557	107.8	1.34
	Cells.	0.648	29.8	0.97	22 20	21.23	1.340	7 270	67.2	

	No. 1	No. 2.	No. 3.	No. 4.
pH$_s$	7.058	7.262	7.433	7.557
$\dfrac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c}$	0.52	0.55	0.53	0.52
$\dfrac{[Cl]_c}{[Cl]_s}$		0.71		0 62
$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	0.91	0.87	0.84	0.82

With these data we have calculated the ratios $\dfrac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c}, \dfrac{[HCO_3]_c}{[HCO_3]_s}$, and $\dfrac{[Cl]_c}{[Cl]_s}$ for oxygenated and reduced blood from pH 7 to 7.6. These

values have been plotted in Figs. 1 to 3 and the interpolated mean values are tabulated in Table X.

TABLE VIII.

Experiment to Determine $\dfrac{[\alpha_{H\cdot}]_e}{[\alpha_{H\cdot}]_s}, \dfrac{[Cl]_e}{[Cl]_s}, \dfrac{[HCO_3]_e}{[HCO_3]_s}$ *in Oxygenated and Reduced Horse Blood.*

Total $[HbO_2]$ = 9.37 mM

No.		$[H_2O]$	CO_2 tension.	$[H_2CO_3]$	Total $[CO_2]$	$[HCO_3]$	$\log \dfrac{[HCO_3]}{[H_2CO_3]}$	pH	$[Cl]$	$[HbO_2]$
		$\dfrac{gm.}{cc.}$	mm.	$\dfrac{mM}{kg.\ H_2O}$	$\dfrac{mM}{kg.\ H_2O}$	$\dfrac{mM}{kg.\ H_2O}$			$\dfrac{mM}{kg.\ H_2O}$	$\dfrac{mM}{kg.\ H_2O}$
1	Reduced, serum.	0.932	155.0	5.058	47.20	42.14	0.921	7.051	106.5	0.23
	Reduced, cells.	0.692	155.0	5.058	44.08	39.02	0.888	6.818	85.4	
2	Reduced, serum.	0.935	42.1	1.372	30.86	29.49	1.333	7.463	112.1	0.83
	Reduced, cells.	0.693	42.1	1.372	26.34	24.97	1.260	7.190	74.8	
3	Oxygenated, serum.	0.936	39.3	1.284	27.63	26.35	1.313	7.443	114.3	9.04
	Oxygenated, cells.	0.668	39.3	1.284	20.13	18.85	1.168	7.098	69.8	

	No. 1, reduced.	No. 2, reduced.	No. 3, oxygenated.
pH$_s$	7.051	7.463	7.443
$\dfrac{[\alpha_{H\cdot}]_e}{[\alpha_{H\cdot}]_s}$	0.58	0.53	0.45
$\dfrac{[Cl]_e}{[Cl]_s}$	0.80	0.67	0.60
$\dfrac{[HCO_3]_e}{[HCO_3]_s}$	0.93	0.85	0.72

Determined ratios have been calculated to 100 per cent oxygenation and reduction by making the following slight correction. The difference between the activity ratios for oxygenated and reduced blood between pH 7 and 7.6 was taken to be a linear function of the pH, approximately represented by the equation

$$r_2 - r_0 = 0.1\ (pH - 6.6)$$

TABLE IX.

Experiment to Determine $\dfrac{[\alpha_{H\cdot}]_e}{[\alpha_{H\cdot}]_c}, \dfrac{[Cl]_e}{[Cl]_c}, \dfrac{[HCO_3]_e}{[HCO_3]_c}$ *in Oxygenated and Reduced Horse Blood.*

Total $[HbO_2]$ = 8.76 mM

No.		$[H_2O]$	CO_2 tension.	$[H_2CO_3]$	Total CO_2.	$[HCO_3]$	$\log \dfrac{[HCO_3]}{[H_2CO_3]}$	pH	$[Cl]$	$[HbO_2]$
		$\dfrac{gm.}{cc.}$	mm.	$\dfrac{mM}{kg.\ H_2O}$	$\dfrac{mM}{kg.\ H_2O}$	$\dfrac{mM}{kg.\ H_2O}$			$\dfrac{mM}{kg.\ H_2O}$	$\dfrac{mM}{kg.\ H_2O}$
	(Reduced).									
1	Serum.	0.934	150.4	4.90	45.22	40.32	0.914	7.044	101.4	0.34
	Cells.	0.712	150.4	4.90	42.86	37.96	0.889	6.819	83.0	
2	Serum.	0.935	66.0	2.15	33.23	31.08	1.160	7.290	105.2	0.49
	Cells.	0.699	66.0	2.15	31.46	29.31	1.133	7.063	74.3	
3	Serum.	0.932	26.1	0.851	23.57	22.72	1.426	7.556	107.2	1.21
	Cells.	0.687	26.1	0.851	19.66	18.81	1.345	7.275	69.6	
	(Oxygenated).									
4	Serum.	0.927	149.4	4.87	41.95	37.08	0 881	7.011	102.8	8.70
	Cells.	0.651	149.4	4.87	38.45	33.58	0.839	6.769	76.8	
5	Serum.	0.933	56.5	1.84	29.78	27.94	1.181	7.311	106.7	7.96
	Cells.	0.655	56.5	1.84	24.26	22.42	1.085	7.015	66.8	
6	Serum.	0.934	24.3	0.79	21.29	20.50	1.414	7.544	110.2	8.72
	Cells.	0.635	24.3	0.79	15.12	14.33	1.258	7.188	62.7	

	No. 1, reduced.	No. 2, reduced.	No. 3, reduced.	No. 4, oxygenated.	No. 5, oxygenated.	No. 6, oxygenated.
pH_s.	7.044	7.290	7.556	7.011	7.311	7.544
$\dfrac{[\alpha_{H\cdot}]_e}{[\alpha_{H\cdot}]_c}$	0.60	0.59	0.52	0.57	0.51	0.44
$\dfrac{[Cl]_e}{[Cl]_s}$	0.82	0.71	0.65	0.75	0.63	0.57
$\dfrac{[HCO_3]_e}{[HCO_3]_s}$	0.94	[0.94]	0.83	0.91	0.80	0.71

The correction, c, applied to the ratio was

$$c = (r_2 - r_0) \times \text{percentage saturation.}$$

Since the correction was in all cases extremely small, no significant error is introduced by the assumption that difference in the activity ratios is a linear function of the pH and of the percentage saturation of the blood with oxygen.

DISCUSSION OF THE DETERMINED DISTRIBUTION RATIOS.

The results, summarized in Figs. 1, 2, and 3, show the following.

(1) The present [HCO₃] concentration ratios (Fig. 1) follow the r curves calculated theoretically by Van Slyke, Wu, and McLean for the blood of their horse with about the same degree of approximation as their [HCO₃] ratios. The present ratios lie above the calculated r curves whereas theirs lay below. The methods used were the same. Consequently the difference in experimental [HCO₃] ratios is presumably due to the fact that the blood used in our experiments differs somewhat in protein or alkali content from theirs. We did not perform total base and protein analyses.

TABLE X.

Summary of Experimental Distribution of H·, Cl, and HCO₃ between Serum and Cells.

pH,	H· activity, Cl molality, and HCO₃ molality ratios.					
	Reduced blood.			Oxygenated blood.		
	$\dfrac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	$\dfrac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$
7.0	0.60	0.81	0.94	0.57	0.74	0.89
7.2	0.57	0.75	0.89	0.52	0.68	0.83
7.4	0.53	0.68	0.85	0.47	0.61	0.77
7.6	0.49	0.62	0.80	0.42	0.54	0.71

(2) The $[Cl]_c:[Cl]_s$ concentration ratios (Fig. 2) are consistently lower than the $[HCO_3]_c:[HCO_3]_s$ ratios. Van Slyke, Wu, and McLean found no consistent difference between HCO₃ and Cl ratios, but stated that the absolute values of their Cl ratios were uncertain, because the Cl method then used was not of tested reliability for concentrated cell substance. We believe that the method here used (3) is reliable, and that the results indicate a genuine difference between the HCO₃ and the Cl molal ratios. Instead of having $\dfrac{[Cl]_c}{[Cl]_s} =$ $\dfrac{[HCO_3]_c}{[HCO_3]_s}$ we have on the average $\dfrac{[Cl]_c}{[Cl]_s} = 0.81\,\dfrac{[HCO_3]_c}{[HCO_3]_s}.$

(3) The hydrion activity values, $\alpha_{H\cdot_s}$ and $\alpha_{H\cdot_c}$, were determined by direct electrometric measurement in reduced blood. In oxygenated

blood the α_{H_c} values were estimated by calculation with Hasselbalch's equation $- \log \alpha_{H^.} = pK_1' + \log \dfrac{[BHCO_3]}{[H_2CO_3]}$. The value used for pK_1', 5.93, was based on electrometric measurements in cell fluids, so that the calculated α_{H_c} values are equivalent to electrometric ones.
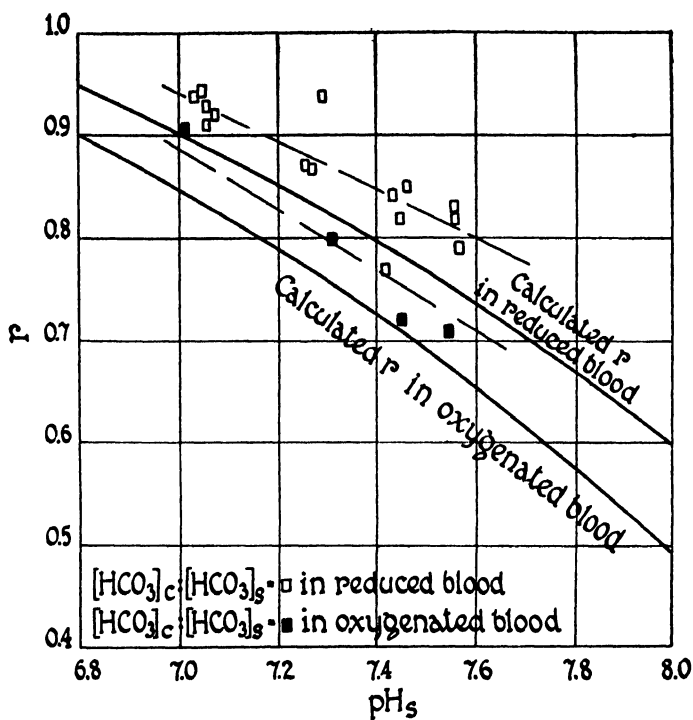
FIG. 1. Points designated □ indicate determined ratios of bicarbonate molal concentrations in cells to bicarbonate molalities in serum at different pH, values o‛ reduced blood. Points designated ■ were obtained from oxygenated blood. The solid curves were plotted from Equation 1.

The activity distribution ratio, $\alpha_{H_c}:\alpha_{H_c}$, showed lower values than the molal $[Cl]_c:[Cl]_s$ and $[HCO_3]_c:[HCO_3]_s$ distribution ratios. The relationship between this fact and the presumable inequality of the activity coefficients in cells and serum will be discussed shortly.

(4) The [Cl] and [HCO₃] molal distribution ratios under the influence of changing pH and oxygenation follow curves which parallel the molar r curves theoretically estimated by Van Slyke, Wu, and

McLean, in their Equations 10 and 14, from the base-binding powers of the proteins (Figs 1 and 2). The observed molal HCO₃ and Cl distribution curves run at definitely different levels from the calculated r curves, the experimental [HCO₃] ratios lying above the theoretical curves, the [Cl] ratios below them. The differences in levels seem not greater than might be expected from the possible effects of the cell and serum constituents on the activities of the two anions.
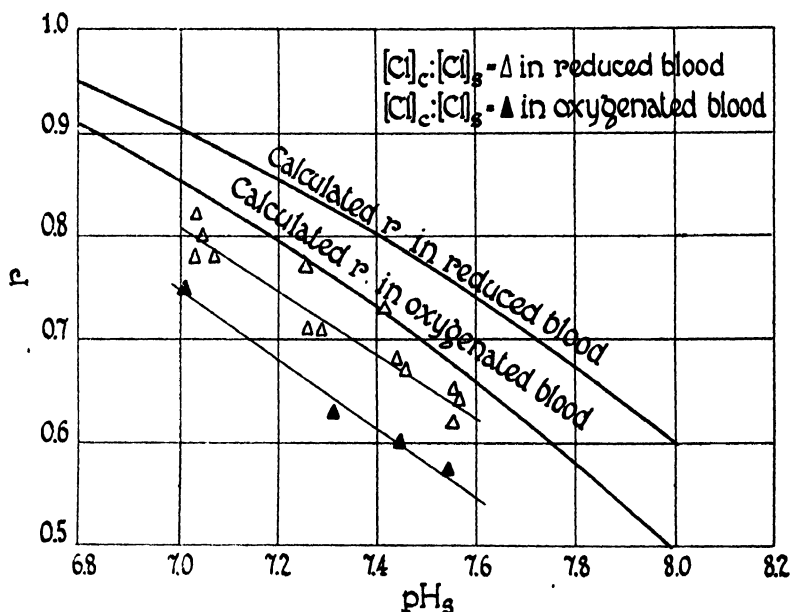
Fig. 2. Points designated △ indicate determined ratios of chloride molal concentrations in cells to chloride molalities in serum at different pH, values of reduced blood. Points designated ▲ were obtained from oxygenated blood. The calculated r curves were plotted from Equation 1.

The electrometrically determined H˙ activity distribution ratios show a similar parallelism with the calculated r curves, although at a much lower level (Fig. 3). The consistent parallelism of observed and calculated effects of both CO₂ and O₂ tension changes on all three ratios affords evidence of the probable adequacy of the physico-chemical laws used to explain the distribution of the diffusible ions as a function of the base bound by the proteins, provided the factors are included which affect the activities of the ions in serum and cells.

Relative Activity Ratios of $H^{\cdot}$, Cl', and HCO_3' in Cells and Serum.

The relationship of our experimentally determined distribution ratios of $\alpha_{H^{\cdot}}$, $[HCO_3]$, and $[Cl]$ may be indicated as follows:

(7)
$$\frac{[\alpha_{H^{\cdot}}]_s}{[\alpha_{H^{\cdot}}]_c} = 0.77 \frac{[Cl]_c}{[Cl]_s} = 0.62 \frac{[HCO_3]_c}{[HCO_3]_s}$$
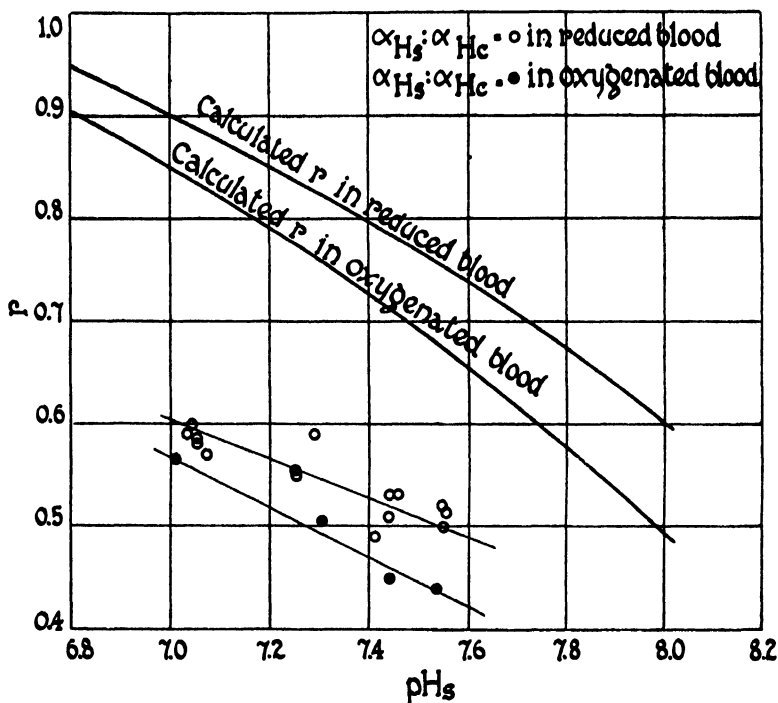
FIG. 3. Points designated O indicate determined ratios of hydrogen ion activity n serum to hydrogen ion activity in cells at different pH_s values in reduced blood. Points designated ● were obtained upon oxygenated blood. The calculated r curves were plotted from Equation 1.

The Cl and HCO_3 concentrations have been determined by accurate chemical analyses, and appear to be trustworthy. It is possible that the high protein content of the cells has affected the E.M.F. readings from which the $\alpha_{H^{\cdot}_c}$ values are estimated. However, we have no independent evidence of such an effect, and the $\alpha_{H^{\cdot}_s} : \alpha_{H^{\cdot}_c}$ ratio is the only one of the three based on methods which theoretically give di-

rectly activity values. Granted the correctness of the α_{H}. values, and of Donnan's law for the thermodynamic activity distribution of the diffusible ions, there must be lower activity coefficients for Cl' and HCO₃' in the cells than in the serum.

This difference necessitates the introduction of activity coefficients into Equations 10 and 14 of Van Slyke, Wu, and McLean, in order to render them exact in indicating activity distributions. It does not, however, affect the validity of the equations in their original form for showing the relationship of the mean *molal* distribution ratios of diffusible anions between cells and serum.

The approximate equality of the *osmotic* activity coefficients of the electrolytes in cells and serum respectively was demonstrated by the chemical analyses of cells and serum reported in Table I of the paper by Van Slyke, Wu, and McLean. It is not at all inconsistent with inequality of the thermodynamic activity coefficients. Assuming equality of the osmotic activity coefficients we may express the osmolar activities of the quantitatively important constituents of cells and serum as follows (see Equation 6 of Van Slyke, Wu, and McLean):

$$(8) \quad 2[BCl]_c + 2[BHCO_3]_c + [BP]_c + [Hb]_c = 2[BCl]_s + 2[BHCO_3]_s + [BP]_s$$

Dividing through by $2([BCl]_s + [BHCO_3]_s)$ we obtain

$$(9) \quad \frac{[BCl]_c + [BHCO_3]_c}{[BCl]_s + [BHCO_3]_s} = 1 - \frac{[BP]_c - [BP]_s + [Hb]_c}{2([BCl]_s + [BHCO_3]_s)} = r_{molal}$$

This is equivalent to Equation 8 of Van Slyke, Wu, and McLean. In this equation no assumption enters of Donnan's activity distribution: the only assumptions concern osmotic pressures, and the conclusion indicated is that the *sum of chloride and bicarbonate* molalities in the cells is related to their sum in the serum as indicated by the right-hand member of the equation. If the Cl molal ratio is greater than the [Cl + HCO₃] molal ratio the [HCO₃] ratio must be less than the latter, and *vice versa*. Such a relation is not excluded. It is in fact the one shown by our present Cl and HCO₃ analyses.

In order to conform with Donnan's law, however, the [thermodynamic activity ratios of the diffusible ions must be equal.

$$(10) \quad \frac{[\alpha_{Cl'}]_c}{[\alpha_{Cl'}]_s} = \frac{[\alpha_{HCO_3'}]_c}{[\alpha_{HCO_3'}]_s} = \frac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c} = r_{activity}$$

Introducing the activity coefficients to indicate the relationship between molality and thermodynamic activity the above equation becomes

$$(11) \qquad \frac{\gamma_{Cl_c} [Cl]_c}{\gamma_{Cl_s} [Cl]_s} = \frac{\gamma_{HCO_{3c}} [HCO_3]_c}{\gamma_{HCO_{3s}} [HCO_3]_s} = \frac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c} = r_{activity}$$

where $[Cl]_c$ etc. represent molal concentrations and γ_{Cl_c} etc. the activity coefficients by which the molalities are multiplied to give thermodynamic activities.

Comparison of Equations 9 and 11 shows that the mean molal distribution ratio r_{molal} of diffusible ions between cells and serum can equal the activity distribution ratio $r_{activity}$ only if the activity coefficient of each diffusible ion is the same in cells and serum; that is,

if $\dfrac{\gamma_{HCO_{3c}}}{\gamma_{HCO_{3s}}} = 1$, etc. Van Slyke, Wu, and McLean, as a preliminary approximation, assumed the existence of this condition. Our present results indicate that the ratios $\gamma_{HCO_{3c}}{:}\gamma_{HCO_{3s}}$ and $\gamma_{Cl_c}{:}\gamma_{Cl_s}$ differ measurably from each other, and that if the electrometric $[\alpha_H\cdot]$ ratios are correct, the γ values for both Cl and HCO_3 are lower in cells than serum.

In Table X are given the experimental data from which are calculated the $\dfrac{\gamma_{HCO_{3c}}}{\gamma_{HCO_{3s}}}$ and $\dfrac{\gamma_{Cl_c}}{\gamma_{Cl_s}}$ values that, when inserted into Equation 11, cause the Cl' and HCO_3' activity distribution ratios thus estimated from the molal ratios, to equal the $\alpha_H\cdot$ distribution ratios. The $[Cl]_c$: $[Cl]_s$, $[HCO_3]_c$:$[HCO_3]_s$, and $[\alpha_H\cdot]_s$:$[\alpha_H\cdot]_c$ values in Table X are obtained by interpolation on the curves representing the mean experimental values in Figs. 1, 2, and 3. The value for $\dfrac{\gamma[Cl]_c}{\gamma[Cl]_s}$ is obtained by dividing each $\dfrac{[\alpha_H\cdot]_s}{[\alpha_H\cdot]_c}$ value by the $\dfrac{[Cl]_c}{[Cl]_s}$ value for the same pH and degree of oxygenation. The eight $\dfrac{\gamma[Cl]_c}{\gamma[Cl]_s}$ values thus obtained ranged from 0.74 to 0.79, and averaged 0.77. Similarly the $\dfrac{\gamma_{HCO_{3c}}}{\gamma_{HCO_{3s}}}$ values were

obtained ranging from 0.59 to 0.64 and averaging 0.62. Equation 11, with these factors inserted, becomes

$$(12) \qquad \frac{\alpha_{H_s^{\cdot}}}{\alpha_{H_c^{\cdot}}} = 0.77 \, \frac{[Cl]_c}{[Cl]_s} = 0.62 \, \frac{[HCO_3]_c}{[HCO_3]_s} = r_{activity}$$

That consistency is introduced among the experimentally obtained distribution ratios by introducing these factors for calculating activity ratios from molal ratios, is demonstrated by the data in Table XI.

<div align="center">

TABLE XI.

Activity Ratios.

</div>

$\alpha_{H^{\cdot}}$ ratios determined by E.M.F. measurement.

$\alpha_{Cl'}$ and $\alpha_{HCO_3'}$ ratios calculated from Cl and HCO$_3$ ratios by arbitrary activity factors.

pH$_s$	Reduced blood.			Oxygenated blood.		
	$\frac{[\alpha_{H^{\cdot}}]_s^*}{[\alpha_{H^{\cdot}}]_c}$	$\frac{[\alpha_{Cl'}]_c\dagger}{[\alpha_{Cl'}]_s}$	$\frac{[\alpha_{HCO_3'}]_c\ddagger}{[\alpha_{HCO_3'}]_s}$	$\frac{[\alpha_{H^{\cdot}}]_s^*}{[\alpha_{H^{\cdot}}]_c}$	$\frac{[\alpha_{Cl'}]_c\dagger}{[\alpha_{Cl'}]_s}$	$\frac{[\alpha_{HCO_3'}]_c\ddagger}{[\alpha_{HCO_3'}]_s}$
7.0	0.60	0.62	0.58	0.57	0.57	0.55
7.2	0.57	0.58	0.55	0.52	0.52	0.51
7.9	0.53	0.52	0.53	0.47	0.47	0.48
7.6	0.49	0.48	0.50	0.42	0.42	0.44

* From pH values directly determined with gas chain.

† Calculated as $\dfrac{[\alpha_{Cl'}]_c}{[\alpha_{Cl'}]_s} = \dfrac{[\gamma_{Cl}]_c}{[\gamma_{Cl}]_s} \dfrac{[Cl]_c}{[Cl]_s}$ with $\dfrac{[\gamma_{Cl}]_c}{[\gamma_{Cl}]_s} = 0.77$

‡ Calculated as $\dfrac{[\alpha_{HCO_3'}]_c}{[\alpha_{HCO_3'}]_s} = \dfrac{[\gamma_{HCO_3}]_c}{[\gamma_{HCO_3}]_s} \dfrac{[HCO_3]_c}{[HCO_3]_s}$ with $\dfrac{[\gamma_{HCO_3}]_c}{[\gamma_{HCO_3}]_s} = 0.625$

If the electrometric pH determinations in cell contents are exact and Donnan's law holds for the distribution of diffusible ions the above γ ratios seem valid. If the cell $\alpha_{H^{\cdot}}$ values should be corrected later, the γ ratios would require multiplication by the same factor and likewise the $r_{activity}$ values, but the soundness of the theoretical considerations on which the later are based would not be affected, nor the

relative proportions of the $\dfrac{\gamma_{HCO_3c}}{\gamma_{HCO_3s}}$ and $\dfrac{\gamma_{Clc}}{\gamma_{Cls}}$ ratios. Thus, if the H$^{\cdot}$

activities should be calculated by Henderson's equation with the same

K_1' values for cells as for serum, the relationships would be expressed as

$$\frac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c} = \frac{[HCO_s]_c}{[HCO_s]_s} - 0.81 \frac{[Cl]_c}{[Cl]_s}$$
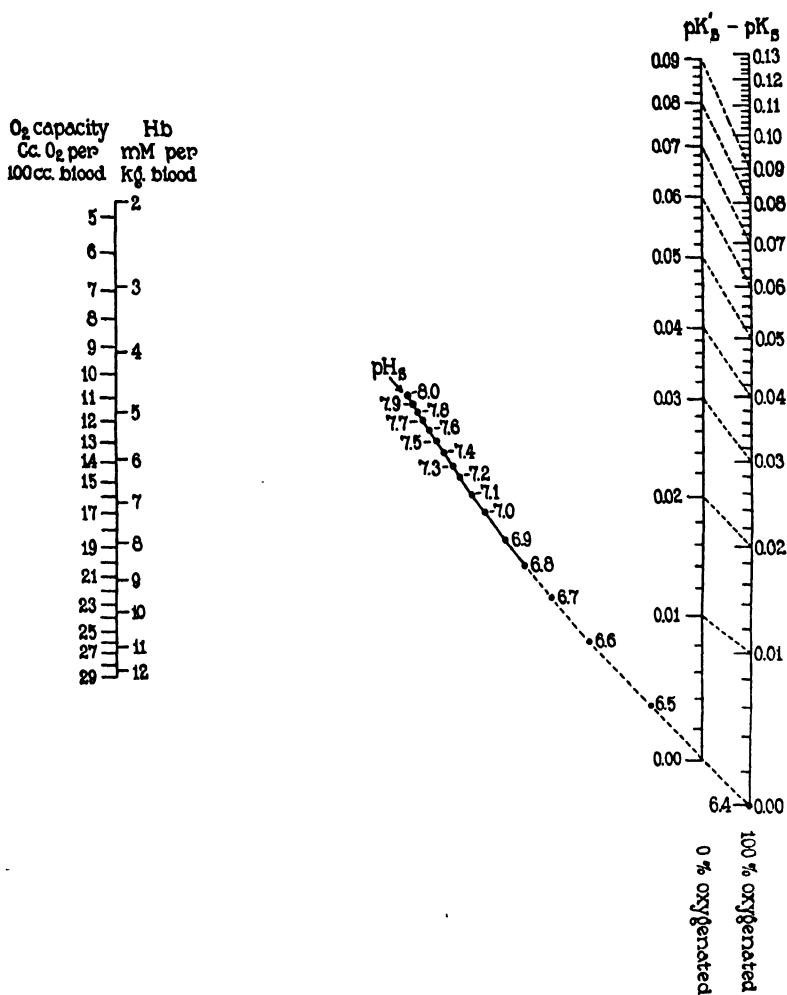
FIG. 4.

We have attempted to obtain independent measurements of the Cl activity coefficients in cells and serum by electrometric α_{Cl} determinations, but the conditions in both cells and serum have thus far prevented the attainment of the necessary accuracy.

Value of pK_1' in Oxygenated and Reduced Whole Blood.

Van Slyke, Wu, and McLean (1) estimated the effect which inequality in HCO₃ distribution has on the pK_1' of Hasselbalch's equation when it is applied to oxygenated whole blood to determine plasma pH. They did not extend their calculation to reduced blood, as the quantitative effect of oxygenation and reduction on the ionic distribution had not then been determined. With the present data in hand such an extension appears now justified, and the results are incorporated in Fig. 4, which is a d'Ocagne nomogram similar to Fig. 6, *b* of Van Slyke, Wu, and McLean, with the addition of a line for the $pK_2' - pK_3'$ values for reduced blood. The dashed slanting lines are to assist in interpolations when pK_1' values are estimated for partially reduced blood.

SUMMARY.

1. The distribution of the diffusible ions, H·, Cl′, and HCO₃′ between serum and cells of horse blood has been studied over the pH range 7.0 to 7.6, and in oxygenated and reduced blood.

2. The experimentally determined distribution ratios have been found to be related as follows:

$$\frac{[\alpha_{H\cdot}]_s}{[\alpha_{H\cdot}]_c} = 0.77 \frac{[Cl]_c}{[Cl]_s} = 0.62 \frac{[HCO_3]_c}{[HCO_3]_s}$$

$\alpha_{H\cdot}$ represents hydrion activity electrometrically determined and [Cl] and [HCO₃] represent molalities, in terms of mols of chloride and bicarbonate per kilo of water.

3. The activity coefficient of HCO₃ in serum estimated from determined [H₂CO₃], [BHCO₃], and pH values was found to be related to the ionic strength in the manner predicted for salt solutions by the theory of Debye and Hückel. For cells, however, the ionic strength as calculated by us indicated a much greater activity coefficient than that found. It appears that in cells unknown factors influence either the activity coefficients or the potential readings obtained with the hydrogen electrode.

4. The changes in the distribution of $\alpha_{H\cdot}$, Cl, and HCO₃ between serum and cells with change in serum pH and in degree of oxygenation

of the hemoglobin approximate those predicted by Van Slyke, Wu, and McLean, from the changes in base-binding power of the cell and serum proteins caused by varying pH and oxygenation.

BIBLIOGRAPHY.

1. Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.
2. Warburg, E. J., *Biochem. J.*, 1922, xvi, 153.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1923–24, lviii, 523.
4. Sörensen, S. P. L., and Linderström-Lang, K., *Compt. rend. trav. Lab. Carlsberg*, 1924, xv, No. 6.
5. Donnan, F. G., *Chem. Rev.*, 1924–25, i, 73.
6. Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, London and New York, 1923.
7. Noyes, A. A., *J. Am. Chem. Soc.*, 1924, xlvi, 1080, 1098.
8. Brönsted, J. N., and La Mer, V. K., *J. Am. Chem. Soc.*, 1924, xlvi, 555.
9. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, 1925, lxv, 445.
10. Hückel, E., *Physik. Z.*, 1925, xxvi, 93.
11. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.
12. Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

THE PROBLEM OF THE ETIOLOGY OF HERPES ZOSTER.

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The nature and etiology of that group of infectious diseases of which one of the features is a vesicular eruption on the skin are at the present time much confused. Rivers (1) has constructed a table indicating a possible relationship between a series of these diseases beginning with sheep-pox and horse-pox and extending through cow-pox, smallpox, varioloid, alastrim, chicken-pox, and herpes zoster to symptomatic herpes and lethargic encephalitis. Certain of these conditions resemble each other in their clinical manifestations, others have little in common. Certainly, the symptoms of herpes simplex have little resemblance to those of smallpox. The only feature present in all of them, except lethargic encephalitis, is a vesicular eruption of the skin. In most of the conditions the skin lesions show similar histological characteristics.

At one time or another some relationship in etiology between various members of the group, or even an identical etiology in all of them has been suggested. In none of the conditions has the etiological agent been cultivated, but there is considerable evidence that the responsible agent in most of them is ultramicroscopic or filterable. It is evident that in the absence of cultivation, in order to establish the etiological relation of an ultramicroscopic virus with one of these diseases, it is necessary to reproduce, in animals or man, lesions resembling the natural infection. With certain of these diseases, notably smallpox and vaccinia, the experimental reproduction of the disease is comparatively easy. With others, such as varicella and herpes zoster, all attempts to transmit them to animals have led only to equivocal and uncertain results. In the case of herpes simplex, although a virus has been isolated which is highly infectious for rabbits, the clinical picture produced is not, as will be discussed below, identical with herpes simplex in man.

Recent investigations have suggested an especially close etiologic relationship between varicella, herpes zoster, and herpes simplex. Some of the workers, mainly on the basis of clinical observations, claim an identity of the etiological agent concerned in varicella and herpes zoster, others are of the opinion that herpes zoster is due to a modified herpes simplex virus.   An etiologic relationship between these conditions is also suggested by the fact that in the epithelial cells of the skin lesions in all of them acidophilic intranuclear inclusion bodies are found.   These bodies were first described by Tyzzer (2) in varicella and later by Lipschütz (3) in herpes simplex and herpes zoster.

Inclusion bodies, both intranuclear and extranuclear, have been studied in great detail by Lipschütz, and are considered by him to be characteristic of diseases of the filterable virus group.   Goodpasture (4) has corroborated and extended Lipschütz's studies of these structures in connection with herpes simplex and agrees with Lipschütz as to the specific nature of intranuclear inclusion bodies. Rivers and Tillett (5) have demonstrated similar intranuclear inclusion bodies in the corium of skin lesions associated with a rabbit virus isolated by them.   In the study of diseases of this group, the finding of these characteristic nuclear changes in experimental lesions in animals or man is therefore of importance in determining whether the reaction obtained is specific.   Following the observation by Grüter (6) in 1920 and those of Löwenstein (7, 8) investigators in all parts of the world have demonstrated that the virus of herpes simplex, when inoculated into the scarified cornea of rabbits produces with great regularity a vesicular eruption followed by an intense keratoconjunctivitis.   Inoculations into the skin less frequently give rise to lesions.   As Doerr and Vöchting (9) first observed, corneal inoculations are frequently followed by marked nervous symptoms and death and similar symptoms can be produced by direct inoculation into the brain.   The inoculations of the virus into rabbits, therefore, gives rise to lesions which *may* resemble those seen in man, but in most cases the lesions and symptoms differ both in character and severity from those present in the mild and common condition in human beings known as herpes simplex.   It is of importance, however, that in all the lesions produced in animals, including those in the cornea, the skin, and the brain, the most characteristic feature of the lesion of herpes simplex in man is reproduced; namely, the occurrence of cells containing intranuclear inclusion bodies.   The intracutaneous inoculation of the vesicular fluid of herpes simplex either into an individual already infected with herpes simplex or into a normal person has not given as constant results as has the inoculation of this fluid into the rabbit's cornea.   Man's susceptibility to herpes simplex seems to depend on certain secondary factors which are, at the present time, unknown.

The successful inoculation of rabbits with herpes simplex material

was followed by attempts to transmit varicella to animals, but so far these attempts have not been successful. (For a review of the literature, see Rivers and Tillett (1).) Many attempts have been made to reproduce varicella in man by inoculating material from active cases into normal individuals. Kling (10) reported the successful vaccination of children against chicken-pox by inoculation with vesicular fluid. Certain later observers employing the method of Kling have noted the development of a local vesicle or papule at the site of inoculation, others have described the occurrence of a generalized eruption (true chicken-pox?), others have stated that no obvious lesions result from the inoculation. So far as we are aware no histological study has been made of any of the lesions described. The difficulty in successfully inoculating animals or man with varicella virus is of interest in view of the claims which have been made regarding the identity of the viruses of chicken-pox and herpes zoster.

Inoculation of Virus of Herpes Zoster into Animals.

Prior to 1921, attempts to inoculate animals with herpes zoster had proved negative. In this year Lipschütz (3) reported successful results.[1] Seven cases which were apparently typical as regards clinical manifestations were studied. The vesicular fluid was obtained early in the disease and was rubbed into the scarified cornea of rabbits. In certain instances the fluid was combined with the "roofs" of vesicles. Lipschütz considers that positive results were obtained with the material from four cases. In view of the importance of his conclusions a brief review of his cases will be given.

Case I.—It is stated that the inoculation of the material into the rabbit's eye was followed in 4 days by a slight opacity of the cornea along the lines of scarification. The eye was removed and sections were made through the cornea. Occasional giant epithelial cells were present along the lines of scarification. Under Bowman's membrane hypertrophied and swollen connective tissue cells

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[1] No attempt will here be made to review the entire literature concerning the inoculation of animals with material from cases of herpes zoster. Attention will be drawn chiefly to those authors whose results have been considered suggestive in contrast to those who have obtained definitely negative results. A complete review of the literature relating to herpes has recently been published by Doerr (34, 25).

were seen but no leucocytes.   In the nuclei of the epithelial cells and also of the swollen connective tissue cells there were to be seen occasional, typical, round, sharply circumscribed and clearly demonstrable intranuclear inclusion bodies.

Cases II and III.—These were also early cases and material from them was similarly inoculated into the eyes of four rabbits.   In one of the rabbits after 4 days the cornea showed an intense circumscribed keratitis, with the appearance of a slightly elevated *"eitrig getrübten,"* and *"daherweiszlich"* appearing vesicle.   The microscopic examination of this eye showed marked infiltration with pus cells. No inclusion bodies were found.   In another animal a keratitis developed, but the occurrence of vesicles or the presence of inclusion bodies is not noted.   The results in the other two animals were negative.   In these two cases, therefore, the evidence presented in the protocols which indicates positive results is very slight.

Case IV.—This was also a typical early case of herpes zoster.   Vesicular material from this case was inoculated into the eyes of two rabbits and two guinea pigs.   The results in the two guinea pigs and in one of the rabbits were negative.   In the second rabbit the inoculated eye showed on the 2nd day conjunctivitis and circumscribed corneal infiltration.   The eye was enucleated on the 4th day and in microscopical sections very numerous intranuclear inclusion bodies were found in the epithelial cells.

The remaining three cases were studied at later periods of the disease and the results were negative.

The experiments of Lipschütz with material from these seven cases can, therefore, as judged from his brief protocols, be considered to have yielded positive results in only two animals, and in these instances the results are of importance chiefly on account of the presence of intranuclear inclusion bodies in the epithelial cells.

Lipschütz (11) considers that his positive findings have been confirmed by Marinesco and Draganesco, Truffi, Mariani, and Blanc and Caminopetros, and that the successful transmission of herpes zoster to animals has thus been accomplished.   It is therefore important to review in some detail the reports of these investigators.

Marinesco (12), and Marinesco and Draganesco (13), injected material from three cases of herpes zoster.

Case I.—Herpes zoster localized on the thigh.   Vesicular fluid was inoculated into the scarified corneas of four small rabbits, and into the second cervical ganglion of two small cats.   The rabbits all remained unaffected.   The ganglia of the cats were examined after 7 days and in one of them there was evident lymphatic infiltration and atrophy of the neurones.

Case II.—Herpes zoster of the first and second branches of the trigeminus nerve. Since the vesicles contained but little fluid, spinal fluid obtained on the 6th day of the disease was used for inoculation.   The injections were made into the anterior

chamber of one eye, and into the scarified cornea of the other eye in each of nine rabbits. Moreover, in five of these nine rabbits, in addition to the eye inoculations, 0.2 cc. of spinal fluid was inoculated intracerebrally. The eyes of the first three rabbits showed only injection due to injury. On the 4th day, in Rabbit 4, in the cornea of the eye in which the injection was made into the anterior chamber, there was noted a zone of infiltration. Rabbit 5 showed two points of infiltration on the scarified cornea. Rabbits 6, 8, and 9 were negative. Rabbit 7, besides a febrile reaction on the 4th day, developed an area of infiltration reaching the center of the pupil. The emulsified brain and cerebellum of this rabbit were inoculated into the cornea of four more rabbits, two of which showed on the 3rd and 4th days grayish infiltration along the lines of scarification. No statement is made concerning microscopic examination.

So far as can be judged from the protocols, therefore, inoculations made with the material from these two cases produced no characteristic lesions. A macroscopic infiltration of the cornea can hardly be regarded as specific.

Case III.—Herpes zoster lesions on the thigh. Vesicular fluid was inoculated into the scarified cornea of three rabbits, two of which on the 4th day showed a linear infiltration. The writer states (12) that the sections of the cornea of one of these rabbits showed swollen, edematous cells, and that here and there could be seen the "specific nuclear lesion, consisting in atrophy of the chromatin which is pushed toward the membrane, while the acidophilic mass, which has developed, offers a striking resemblance to the inclusions described by Lipschütz in animals injected with herpes." It is undoubtedly on this last statement that Lipschütz bases the view that his own observations have been confirmed by Marinesco and Draganesco. Certainly nothing else in the protocols indicates the occurrence of a specific lesion.

Truffi (14) studied three cases of herpes zoster. The results in the first two were negative. Vesicular fluid from the lesion of Case III, cervicobrachial in distribution, was obtained on the 3rd day of the disease and inoculated into the scarified cornea of one rabbit. After 48 hours a slight opacity along some of the lines of scarification, and an intense conjunctivitis were noted. The corneal opacity disappeared rapidly and the eye regained its normal appearance by the 7th day. 22 days after inoculation the rabbit showed symptoms of encephalitis and was killed 10 days later. The microscopical examination of the brain was negative. The presence of intranuclear inclusion bodies in the brain cells is not noted. The inoculation of the brain emulsion into the scarified corneas of two rabbits and two guinea pigs failed to produce lesions.

Most of the attempts made by Mariani (15) to inoculate the cornea of rabbits with herpes zoster resulted negatively. In one instance he obtained a very acute keratoconjunctivitis with hypopyon and purulent ophthalmia. In only one case did there result a keratitis which he was able to transmit in series. The lesion produced was clinically and symptomatically very similar to the keratitis produced by herpes simplex virus. No description of the case of herpes zoster from which

the material for inoculation was obtained, is given. No statement concerning microscopic examination of the corneas is made. Mariani himself considers this single experiment inconclusive.

Material from nine cases of herpes zoster was inoculated by Blanc and Camino-petros (16) into the eyes, cornea, conjunctiva, skin, brain, and spinal cord of a series of animals, including rabbits, mice, sheep, pigeons, monkeys, and a dog. Three monkeys (*Macacus rhesus*) were inoculated as follows: one into the eye, one into the spinal canal, and the third into the skin of the thoracic region which had previously been shaved and excoriated. The inoculations in the first two monkeys resulted negatively. The third monkey showed a slight inflammatory reaction at the site of inoculation but recovered without the appearance of vesicles. All the other animal experiments gave negative results with the following exceptions; two rabbits developed a late paralysis which, however, the authors considered was probably not specific, and one rabbit and one sheep, both inoculated with material from the same case, developed a definite keratitis, which spread from the point of inoculation. The writers think that this lesion might easily be interpreted as a reaction resulting from the injection. They conclude that the problem of the transmission of herpes zoster to animals remains open and they apparently consider their own experiments negative or inconclusive.

Meineri (17) claims to have produced encephalitis in a guinea pig by the intra-cerebral inoculation of vesicle fluid from a case of herpes zoster. A careful analysis of his experiments, however, in our opinion, indicates that his findings can best be interpreted as the result of trauma. The writer also injected vesicle fluid obtained on the 3rd day of the disease from one of his cases of herpes zoster into the skin of the arm of the patient and into the skin of a normal man. These injections in both instances were without visible result.

The review of the publications of those writers whom Lipschütz quotes as having confirmed his work shows that two of the writers regard their own results as inconclusive. Only Marinesco and Drag-anesco found microscopical lesions which might be interpreted as specific.

On the other hand, many other authors report entirely negative results following the inoculation of herpes zoster material into the sacrified corneas of rabbits: Kraupa (18); Baum (19); Löwenstein (8); Teissier, Gastinel, and Reilly (20); Kooy (21); Netter and Urbain (22); Bloch and Terris (23); Simon and Scott (24); and Doerr (25).

It is evident, therefore, that the results of attempts to inoculate animals with material from cases of herpes zoster must be considered at present to be inconclusive.

Herpes Simplex and Herpes Zoster.

Although it has not been possible to demonstrate conclusively any specific virus associated with herpes zoster, certain writers have presented evidence which suggests that, in certain cases at least, the symptoms and lesions of herpes zoster may result from the presence of the virus of herpes simplex.

Luger and Lauda (26) have published several papers on the problem of the etiology of herpes zoster. In their first paper they give the results obtained by inoculation with material from seven cases of typical herpes zoster, employing the technique used by Lipschütz. In none of the eyes inoculated did any macroscopic reaction occur. On microscopical examination there was found fairly regularly edematous swelling of the epithelial cells, giant cell formation, and *"ballonierende"* degeneration, but in no instances were cell inclusions or characteristic changes of the nuclei seen. They themselves considered the results in this series of experiments negative. This paper was followed by two others (26) in which two more cases, considered by the authors to be typical cases of herpes zoster, were studied. From both of these latter cases these investigators were able to isolate a virus, infectious for rabbits, which they showed, by immunity experiments, to be identical with the virus of herpes simplex. The first case occurred in a patient suffering from acute epidemic encephalitis, and the eruption was localized in the gluteal region. The involved area was not large and the clinical diagnosis of herpes zoster in this case must be considered doubtful. A typical herpes simplex virus was isolated without any difficulty. In the second case the eruption was thoracic in distribution and the case was clinically typical. On the 8th day of the disease, some vesicle fluid was inoculated into the eye of one rabbit. No local reaction occurred. The 10th day after inoculation the animal developed encephalitis and died on the 11th day. For reasons not stated, the glycerolated brain of this animal was stored on ice for 11 days before further inoculations were made. Inoculations then made with this material into the brains and eyes of rabbits produced the characteristic reactions of a herpes simplex virus. The authors conclude from these results that herpex simplex virus may be isolated from herpes zoster vesicles. They offer no explanation of the fact that although the virus failed to produce any reaction on the cornea, it nevertheless possessed the ability to invade the brain.

Grüter (27) inoculated material from three cases of herpes zoster into the scarified corneas of rabbits. A mild keratitis resulted. No detailed description of the lesion or results of microscopic examination are given. Grüter, however, believes the lesion obtained was specific and attributes it to herpes simplex virus of a low grade of virulence. He states that there is no evidence for assuming a specific virus for herpes zoster. The data presented, however, are not sufficient to establish the isolation of a true herpes simplex virus from these cases.

Bastai and Busacca (28), in a general article on herpes, state that they inoculated material from three cases of herpes zoster into the cornea of rabbits and into the cornea of one monkey (*Macacus*).   Rabbits were also inoculated intracerebrally.   None of the animals showed any reaction, with the exception of one rabbit which developed a slight keratitis.   No attempts were made to transmit this lesion, and no microscopical examinations are reported.   These authors also are of the opinion that herpes zoster is probably a manifestation of infection with herpes simplex virus.   The experimental data presented, however, are hardly sufficient to justify this point of view.

Cipolla (29) is of the opinion that the cases of herpes zoster should be divided into two groups, zoster symptomaticus and zoster idiopathicus, although he states that there is great difficulty in drawing any sharp line of division.   He has studied four cases which he considered zoster idiopathicus and three cases which he called zoster symptomaticus.

The results of inoculation of material from the so called idiopathic cases were as follows: In one rabbit and two guinea pigs there occurred "a keratitis definitely evident along the lines of scarification but without vesicle formation and a hyperemic conjunctivitis, with redness and slight seromucous exudation."   The results in all the other animals inoculated were negative.   No note is made of any microscopical examinations.

Of the cases which he considered symptomatic, in one the eruption followed an injury, in the second it was associated with a tuberculous pleurisy, and in the third the patient was taking bromides and the herpetic eruption was considered to be a manifestation of bromide intoxication.   In all these cases the eruption was on the chest.   In two cases the appearance of vesicles was preceded by acute neuralgic pains, in the third case no note is made concerning pain, but the eruption was accompanied by a febrile reaction.   The inoculations with material from all these cases gave rise to severe keratitis and conjunctivitis resembling that caused by the virus of herpes simplex.   In one rabbit an encephalitis developed.   No statement concerning microscopical examinations is made.

In our opinion the three so called symptomatic cases must be considered from the clinical features as probably cases of herpes zoster.   The comparatively trivial associated injuries (trauma or drug) might easily have been overlooked and then the diagnosis would not have been questioned.   It is of great interest that the animal experiments indicate that in these cases a virus indistinguishable from that of herpes simplex was present in the lesions.

Teague and Goodpasture (30) were able to produce zoster-like lesions in the skin of rabbits and guinea pigs by the inoculation of herpes simplex virus into areas of the skin previously treated with coal tar.   The study of the corresponding posterior root ganglia showed lesions comparable to those found in man in the ganglia innervating the area of zonal eruption.

The writers do not maintain that they have reproduced the human disease herpes zoster in animals, but they believe there is a close analogy between the experimental condition produced by them and true herpes zoster.   In their

opinion the herpes simplex virus first multiplies at the site of inoculation in the skin and passes up the corresponding spinal nerve to its spinal ganglion; the virus then seems to pass centrifugally along the nerve and its branches to the skin, where it multiplies rapidly and gives rise to characteristic herpetic vesicles. They draw attention to the difficulty of sharply separating cases of herpes simplex and herpes zoster and discuss the occurrence of intermediate cases. From a case of the intermediate type inoculations were made into the tarred skin of a rabbit. A zonal eruption, as described above, resulted. They feel that the evidence presented strongly suggests that the virus of herpes zoster is closely allied to the virus which causes herpes simplex, probably differing only in virulence.

The interesting hypothesis presented by Teague and Goodpasture (30) and by Luger and Lauda (26) concerning the relation of herpes zoster to herpes simplex does not find acceptance, however, by Lipschütz (31). He emphasizes the point of view that in the production of an experimental herpes zoster it is of prime importance that the starting point be a typical clinical case and not a border line case.

At the present time the evidence that herpes zoster may result from infection with herpes simplex virus rests upon the isolation of a virus apparently identical with that of herpes simplex from a small number of cases. No description of the type of case from which the material employed for inoculation was obtained is given by Grüter or by Bastai and Busacca. The case described by Teague and Goodpasture and the first case described by Luger and Lauda belong to the intermediate type of cases. The second case of Luger and Lauda, and the so called symptomatic cases of Cipolla, seem to have been clinically typical cases of herpes zoster. It is possible, therefore, that in certain instances the virus of herpes simplex may be isolated from cases clinically characteristic of herpes zoster, but the evidence for this is not complete and the conclusion that herpes zoster may be the result of infection with herpes simplex virus needs further verification.

Varicella and Herpes Zoster.

Several observers (Lipschütz, Mcineri, and others) have made isolated attempts to inoculate human volunteers with herpes zoster, but always with negative results. Recent studies of Kundratitz (32) seem to show that herpes zoster can be successfully transmitted to very young children. This author wished to test out von Bókay's (33) hypothesis, based on clinical observation, that the virus of varicella, under certain unknown conditions, may produce a typical picture of herpes zoster and that the virus from this lesion may in turn cause varicella. He therefore

attempted to immunize children against varicella by the inoculation of material from herpes zoster cases. His first results were negative, but his later attempts proved successful. He now reports that he has inoculated material from ten typical cases of thoracic herpes zoster and has had positive results with the material from five of these cases. Positive reactions were obtained only in children under 5 years of age. Children who reacted positively were subsequently shown to be immune to varicella.

Kundratitz's work seems to indicate that the virus of varicella and that of herpes zoster are identical or, at least, closely related. It is unfortunate that Kundratitz does not give a description of the cases of herpes zoster used by him for inoculation. It would be interesting to know whether there were any clinical differences between the five cases of herpes zoster with which he was able to make successful transfers and the five cases in which transfers resulted negatively for, as von Bókay and others have shown, the vesicles of varicella may be quite localized, resulting in lesions resembling herpes zoster. The relation between herpes zoster and varicella will, in all probability, not be entirely cleared up until we are able to transmit either one or both of these diseases to animals.

EXPERIMENTAL.

Nine cases of herpes zoster have been studied by the writers and inoculations have been made into a series of animals. The following are abstracts of the histories and protocols of the experimental studies.

Case I.—A. M. Age 13. Patient admitted to the hospital Oct. 4, 1924, suffering from subacute rheumatic fever and chronic cardiac disease. She gave no history of a previous attack of herpes zoster or chicken-pox. The arthritis had almost entirely disappeared and the cardiac lesion was well compensated, when on Dec. 6, 1924, the patient complained of pain and itching over the upper scapular area, in the axilla, and posterior part of the upper arm. On examination of this area there was discovered a rash consisting of small, discrete papules and vesicles distributed in patches over a zone on the upper chest from the midline behind to the midsternal line in front, and over the inner and posterior surface of the arm. The area of distribution corresponded to Head's second and third dorsal areas. During the following days the vesicles became larger. The temperature was not higher than 99.8° until Dec. 11, when some of the vesicles had become pustular, and now the temperature rose to 101.4°. The pain was severe and characteristic of herpes zoster, and the appearance and distribution of the lesions were typical. A small piece of skin was removed and microscopical sections showed characteristic vesicles with numerous intranuclear inclusion bodies in the epithelial cells.

On Dec. 9, the 3rd day of the disease, fluid was pipetted from a number of vesicles and a small piece of the involved skin was obtained. The skin was ground between two glass slides, the ground material was washed off in a small amount of normal saline solution and was added to the vesicular fluid. Small amounts of this emulsion were rubbed into the scarified[2] corneas of two rabbits, Nos. 1 and 2, and also injected intracutaneously into the shaved skin of Rabbit 2 and into the skin of Guinea Pig 1. The area of skin in the guinea pig where the injection was made had been painted several days previously with coal tar solution. The emulsion was also rubbed into the scarified skin of Rabbit 3, which had received one painting of tar 5 days before, and into the scarified skin of a similarly tarred guinea pig, No. 2. (The rabbit and guinea pigs were painted with a refined coal tar solution obtained through the courtesy of Dr. Jas. B. Murphy. This refined coal tar was much less toxic than ordinary tar and could be applied in a fairly thick coat, so that one painting resulted in a marked reaction.) On the following day, Dec. 10, vesicular fluid was again obtained from fresh vesicles and also another piece of skin. This material was treated in the same way as that obtained on the preceding day, and inoculated in the following ways. The cornea of Rabbit 1 and the scarified tarred skin of Rabbit 3 and Guinea Pig 2 were reinoculated. Some of the material was also inoculated intracerebrally into Rabbit 4 and into the scarified cornea of Rabbit 5. Thus, with fresh material obtained on the 3rd and 4th days of the disease, five rabbits and two guinea pigs were inoculated in various ways.

The animals were carefully observed each day following the inoculations, but in none of the animals were any macroscopic changes seen which could be ascribed to the inoculations. One of the eyes of Rabbit 2 was removed on the 3rd day and the other on the 7th day following the inoculations and sections were made through the corneas. The sections show in places what are apparently the results of mechanical injuries and in the section of the eye removed on the 7th day, foci of slight infiltration of the substantia propria with small round cells. Some swelling of certain of the epithelial cells is also seen. But nowhere are there any signs of vesicle formation or marked inflammatory reaction and no inclusion bodies were found.

Although no definite reactions were obtained in this first series of animals it was thought that by inoculating from one cornea to another and from one brain to another, the virus might possibly become adapted to the rabbit and produce definite lesions in subsequent transfers. Therefore, starting with Rabbit 1 inoculations were made from one rabbit to another by scraping the cornea and washing out the conjunctival sac with a small amount of saline and inoculating the material thus obtained into the scarified cornea of another rabbit. Fourteen corneal passages were thus made, at 2 and 3 day intervals. In many of the rabbits, the scarified eye on the day following the inoculation showed a slight degree of opacity along the lines of scarification and a slight exudation. However, it was found

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[2] The eyes of the rabbits were anesthetized locally with cocaine before inoculation. For other operations the animals were given ether.

during the course of the study that slight changes of this character frequently occur following the inoculation of an emulsion of normal rabbit cornea, and even after scarification alone without the injection of any foreign matter whatever. Except for these slight non-specific reactions no changes were observed in any of the eyes of the series. In certain instances, although no gross changes were present, the cornea was sectioned but no lesions which could be considered specific and no intranuclear inclusion bodies were found.

Starting with Rabbit 4 inoculated intracerebrally with the material from this case, ten brain to brain transfers were made at 5 day intervals. Each animal was killed with ether, and the brain removed with sterile precautions. An emulsion of the brain was made with Locke's solution in a sterile mortar, the suspension centrifuged at low speed, and 0.2 cc. of the supernatant fluid injected intracerebrally into a normal animal. At the same time, some of the brain emulsion was inoculated intracorneally and intradermally into each of two other rabbits. It was found that the inoculation of brain emulsion into the scarified cornea usually was followed by conjunctivitis of considerable severity which, however, proved to be wholly non-specific. The temperature of the intracerebrally inoculated rabbits was taken daily, and sections of the brain of each of the inoculated animals made. None of the rabbits showed a significant rise in temperature and careful study of the brain failed to reveal any characteristic lesions. No intranuclear inclusion bodies were found.

Case II.—F. T. Age 13. Patient admitted to the hospital Nov. 13, 1924, suffering from rheumatic fever and cardiac disease. History unimportant in the present connection except that the patient stated that he had had chicken-pox. On the afternoon of Feb. 1, 1925, the patient showed a definite rise in his pulse rate (120). This was attributed to the excitement due to visitors and playing a game. There was no rise in temperature. On Feb. 2 there was discovered a vesicular (herpetiform) eruption over a small area on the lower-right chest midway between the nipple and the costal margin. He did not complain of pain or itching and there was no constitutional disturbance. On Feb. 4 when the vesicles were already beginning to become dry, the patient complained of slight itching. A small piece of skin where the rash was present was removed at this time. A portion of this was cut into sections and showed characteristic multilocular vesicles, with swollen epithelial cells containing intranuclear inclusion bodies.

This patient was in the hospital at the same time as Patient I. The two patients were at opposite ends of the same floor, but in different wards. Case II developed nearly 2 months after Case I, and it is not likely that there was any direct connection between them.

The lesion in Case II was so slight that it was thought the condition might be that described in the literature as zosteriform herpes rather than true herpes zoster. The distribution of the vesicles was suggestive of herpes zoster, but the vesicles remained small, the patient never complained of pain in the affected area, and there was no fever.

In view of the clinical course in this case and the somewhat uncertain diagnosis

it was decided to inoculate all the material obtained into the corneas of rabbits. If the lesion were due to herpes simplex virus which had accidentally become implanted on the thorax, the inoculation of the material from this case into the scarified corneas of rabbits should produce typical keratitis. If, on the other hand, the condition were true herpes zoster, it would be advisable to employ as nearly as possible the technique used by Lipschütz. Consequently the piece of skin removed from the affected area was ground up in a mortar without sand in a small quantity of normal saline solution. Portions of the suspension were then rubbed into the scarified right corneas of six young rabbits. Cocaine was used instead of ether to avoid lacrymation. After inoculation the eye was held in position for a short time so that the inoculum might dry on the cornea. The left cornea of each animal was scarified in the same way as the right and left uninoculated to serve as control.

Daily comparisons were made between the inoculated corneas and the corneas which had merely been scarified. In some of the animals a slight conjunctivitis was observed in one or the other eye. But whereas in one instance the reaction seemed more intense in the inoculated eye, in another the control eye appeared worse. In none of the animals did a severe keratitis develop. The possibility that the infective agent in this case might be herpes simplex virus was, therefore, in all probability excluded.

On the 4th day after inoculation although no definite macroscopic lesion developed, the right eyeball of each of the six rabbits was removed, fixed in Zenker's solution, and sectioned. Careful examination of the sections of the six corneas of the inoculated rabbits failed to show any definite lesions or the presence of intranuclear inclusion bodies.

Case III.—J. L. Age 16. The patient was admitted to the hospital Oct. 5, 1924, suffering from diffuse glomerular nephritis. The patient stated that neither he nor any other member of his family had had chicken-pox. On Feb. 11, a vesiculopapular eruption appeared on the lateral and anterior aspects of the left side of the lower chest and upper abdomen, extending over the lumbar region to the midline behind. The eruption occupied the region corresponding to Head's eighth dorsal area and was typically that of herpes zoster. It is of interest that in this patient there appeared on Feb. 17, on the right or opposite side, over the abdomen and flank, a vesicular eruption. The eruption here was exactly like that on the opposite side but much less extensive, and also had a zonal distribution, but slightly lower than on the left, corresponding to Head's tenth dorsal area. Bilateral distribution of the rash in herpes zoster is rare though it does occur, as in this case. This patient occupied a bed directly opposite that of F. T., Case II, and the rash first appeared just 10 days after that of F. T. As herpes zoster is extremely uncommon in this hospital it seems reasonable to suspect some connection between these two cases and also some relation between these cases and Case IV. On the day after the rash appeared a small piece of skin where the rash was present was removed and a little vesicle fluid was obtained. At this time most of the rash was still in the papular stage. A portion of the skin was sectioned and the

examination showed the typical lesions of herpes zoster with many cells containing intranuclear inclusion bodies.

The skin removed on Feb. 12 was ground up in a mortar with Locke's solution and combined with the vesicle fluid. The material was centrifuged at low speed for a few seconds to throw down the larger particles, and about 1.5 cc. fluid was obtained. Monkey 1 (*Macacus rhesus*) was inoculated with this fluid in the follow-ing ways: intracerebrally, intracutaneously, into the scarified cornea, and directly into the anterior crural nerve. No reactions occurred at the sites of the corneal and intracutaneous inoculations. No nervous or other symptoms developed. The monkey later developed tuberculosis and was killed on Mar. 9.

On Feb. 14, 1925, the 3rd day of the disease, the "roofs" of four more vesicles were removed, ground up without sand, and inoculated into the scarified corneas of six rabbits. Some of the same material was also inoculated into the scarified skin on the arm of a human volunteer whose history was negative for chicken-pox and herpes zoster. The result of the latter experiment was negative. Three of the inoculated rabbits were bled to death after 24 hours, the eyeballs removed and fixed in Schaudinn's solution and stained with Giemsa. The other three rabbits were observed for 72 hours, the eyeballs removed and treated in the same way. The sections of the corneas of these six rabbits showed no lesions other than those resulting from the scarification. No intranuclear inclusion bodies were found.

Case IV.—R. N. Age 37. This patient developed the lesions of herpes zoster on Feb. 22, 1925. He occupied a bed in the same ward as Patients II and III, and the rash appeared 11 days after that of Case III. The patient was an adult man, 37 years of age. He was admitted to the hospital on Dec. 12, 1924, suffering from diffuse glomerular nephritis. He stated that as far as he knew he had never had chicken-pox. On Feb. 22, 1925, a vesicular eruption appeared over a zone extending over the right lumbar region posteriorly and just below the umbilicus anteriorly. The area of distribution corresponds roughly to Head's tenth dorsal area. The rash was not preceded by any subjective symptoms and was not no-ticed by the patient until pointed out to him by another patient. The patient seemed quite comfortable on this date. On Feb. 23 the eruption became more marked, and groups of large, tense vesicles were present. Considerable hyper-esthesia developed in the involved area. The patient's temperature rose to 101.6° and he complained of general malaise. On the following days he complained of pain, both superficial and deep, along the course of the involved nerve root. The deep pain was of a sharp, cutting character, and paroxysmal in type, and was referred to the right iliac region. Some of the larger vesicles later became hemor-rhagic and an enlarged, very tender axillary lymph node appeared on the affected side.

On Feb. 24, the 3rd day of the disease, some vesicle fluid was collected in a sterile syringe containing a trace of sodium oxalate solution. The fluid was injected into the spinal cord of Monkey 2 (*Macacus rhesus*). The monkey was shaved over the dorsal region on both sides so that in case a rash developed it would be

visible. Some of the vesicle fluid clotted before all the injections could be made and the clot was placed in a mortar and ground up with a small amount of salt solution. This material was injected intracerebrally and intracutaneously into Monkey 2, and a small amount was also rubbed into the scarified cornea. The results of the skin and eye inoculations were completely negative. No cutaneous reactions appeared at the level of the spinal cord inoculation. The monkey never showed any nervous or other symptoms. No rise in temperature was observed.

On Mar. 28, a month after the inoculation with herpes zoster material, the monkey's susceptibility to herpes simplex virus was tested by intracerebral inoculation of a virulent herpes simplex virus. The monkey never showed a febrile reaction or any other symptoms.

On Feb. 24 vesicle fluid from the patient was obtained and immediately rubbed into the scarified cornea of a rabbit. The rabbit showed a slight amount of conjunctivitis after 24 and 48 hours, but the eye appeared normal after 72 hours.

On Feb. 28 the "roofs" of four vesicles were removed. This tissue was ground without sand, mixed with a small amount of saline, and the suspension was injected intracranially into two rabbits and intraspinally into two others. The rabbits which received the injections into the cord were shaved at the level of inoculation so that any skin reaction would be visible. All four of the rabbits remained well, and never showed any febrile or other reaction.

Case V.—E. K. Age 19. Student. The patient was admitted to the hospital May 23, 1925, with the diagnosis of herpes zoster. The past history is unimportant except that he is sure that he had chicken-pox at the age of 5. On May 18, 5 days before admission, he first noticed a slight dull pain in his chest on the left side, and this pain persisted. On May 20 the pain became much worse, and an eruption of "small blisters" over the left breast, anterior to the axilla, was noted. He had slight fever and slight general malaise. He described the pain as being a "stinging itch;" sometimes it became a dull, constant pain. The pain was present not only over the area involved in the rash, but radiated to the lower left back. On admission there was present on the left side of the thorax an irregularly arranged, rather sharply delimited, papulovesicular eruption. The eruption occupied, roughly, a horizontal zone 6 to 8 cm. broad, just below the level of the nipple, occupying Head's fifth dorsal area. The vesicles varied in size, and were mostly discrete. There was slight tenderness over the affected area and there was some hyperesthesia of the uninvolved skin in this area. A small piece of the affected skin was removed on May 23. Microscopical sections of this showed typical vesicles of herpes zoster with numerous intranuclear inclusion bodies. At the same time three other small pieces of skin were obtained and ground without sand in a sterile mortar in a small amount of Locke's solution. 0.2 cc. of the supernatant fluid of this emulsion was injected into each testicle of a young vervet monkey (*Cercopithecus lalandii*), commonly called the green monkey. The monkey's temperature remained normal on the succeeding days. On the 4th day after inoculation the left testicle was removed. No gross changes were evident. The whole testicle was placed in Zenker's solution and the following day was divided

into five blocks and sections were cut from each block. On the 5th day after inoculation the right testicle was removed and treated like the left. Numerous sections from both testicles were studied and, although the left testicle seemed to show a definite lesion with hemorrhage and necrosis, no typical intranuclear inclusion bodies were found in sections from either testicle.

The attempt to inoculate a vervet monkey with material from this case was, therefore, unsuccessful. There is still disagreement among the various investigators as to whether monkeys are susceptible to herpes simplex virus. An attempt was made to infect this monkey later with fresh virulent herpes simplex virus but no reactions occurred. This species of monkey is therefore probably insusceptible or at least not highly susceptible to herpes simplex virus.

Case VI.—P. C. Age 41. The patient was admitted June 30, 1925. He was uncertain whether or not he had had chicken-pox. On the evening of June 25, 1925, when the patient was walking home from work he felt an intense itching sensation around the right scapular region, extending anteriorly around into the right axillary region. Later in the evening small vesicles began to appear in this area. The rash appeared over the right scapula and anteriorly below the right mammary region, the distribution corresponding to Head's third and fourth dorsal areas. There was considerable pain in the involved regions.

On June 30, on the 6th day of the disease, four small pieces of skin were removed and ground in a sterile mortar without sand in a small amount of Locke's solution. 0.1 cc. of this emulsion was injected into each testicle of a young vervet monkey. On the following day, July 1, 1925, the monkey's temperature showed no striking rise. Both testicles were enlarged. 24 hours after inoculation the left testicle was removed. The whole testicle was placed in Zenker's solution, and the next day was divided into three blocks. July 2, the right testicle was removed and treated in the same way. Numerous sections from both testicles were studied, but no specific lesions with intranuclear inclusion bodies were found.

Case VII.—J. S. Age 38. The patient was admitted July 2, 1925. The patient has syphilis and had received antisyphilitic treatment at the Presbyterian Hospital since March. She did not know whether or not she had had chicken-pox. For 2 or 3 days before admission she had pain over her left breast and over the left side of her back in the scapular region and in her left arm. On July 1, small vesicles appeared on her back and on the left breast, and a fairly large crop of vesicles appeared in the axillary region. The distribution corresponded to Head's fourth dorsal area.

On July 2, 1925, the 2nd day of the disease, some vesicle fluid was collected from the larger vesicles, and seven small pieces of skin were removed and ground up without sand in a small amount of Locke's solution. The vesicle fluid was added to the skin emulsion, and 0.2 cc. of this material was injected into each testicle of a young vervet monkey. An intracutaneous injection was also made. The following day the testicles appeared enlarged. 40 hours after inoculation the right testicle was removed and sections made. 72 hours after inoculation the left testicle was removed and sections made. Numerous sections of both

testicles were studied, but no specific lesions with intranuclear inclusion bodies were found. The result of the intracutaneous inoculation was completely negative.

Inasmuch as this patient was receiving injections of salvarsan and mercury, it was thought that this case, in spite of the typical clinical course and characteristic distribution of the vesicles, might represent an example of so called symptomatic herpes zoster described particularly by Cipolla and in the cases of which he found the virus of herpes simplex. Tests were therefore made for the presence of herpes simplex virus by inoculating some of the material from this case into the eyes of four rabbits, but the results were negative.

Case VIII.—S. B. Age 14. The patient was admitted July 13, 1925. The patient had been in the hospital since May 22 suffering from pleurisy. She stated definitely that she remembered having had chicken-pox at the age of 6 or 7 years. On July 12 she complained of pain in her right shoulder and on examination a vesicular rash covering the right scapular region was found The eruption extended from the lower half of the wing of the scapula to the vertebral column. A few isolated vesicles were present anteriorly to the right of the nipple. The area involved corresponded to Head's fourth dorsal segment. A slight hyperesthesia of the skin was noted.

On July 14, the 3rd day of the disease, three fairly large pieces of the involved skin were obtained and ground up without sand in a small amount of Locke's solution. Injections of this material were made in the following ways. It was injected into the testicles of a young vervet monkey, intracutaneously into the scarified skin of one tarred guinea pig and one tarred rabbit, and into the scarified corneas of two rabbits. The inoculated eyes and skin remained completely negative. On July 15 the monkey's temperature was 103°. On July 16 the temperature was 100.6° and the right testicle was removed for section. On July 17 the left testicle was removed. Numerous sections of both testicles were studied but no specific lesions with intracellular inclusion bodies were found.

Case IX.—H. S. Adult male. The patient had had chicken-pox in childhood but had never had herpes zoster and had not recently been in contact with any case of herpes zoster. His wife had had a herpes simplex eruption a short time previously. The papular rash over the right shoulder appeared after 2 weeks of slight indisposition and after several days of severe headache and pain in the back.

On the 1st day of the eruption the tops of three papules were removed, immediately ground without sand, and injected into both testicles of a young vervet monkey. There occurred no febrile reaction and on the 3rd day the left testicle was removed and on the 4th day the right testicle was removed. Evidences of injury with hemorrhage were seen but on section no marked cellular reactions were present and no inclusion bodies were found. Some of the material from this case was also inoculated into the scarified corneas of two rabbits with negative results.

SUMMARY AND DISCUSSION.

Attempts were made to produce lesions in animals by the injection of material obtained from the vesicles and involved skin of nine cases of herpes zoster. All the cases, with the exception of one (Case II), were characteristic cases of idiopathic herpes zoster and the question of their being cases of so called zosteriform herpes or symptomatic herpes zoster can hardly be raised. As regards Case II, if this case occurred alone, there might be some doubt as to its nature on account of the mildness of the symptoms and the small area of skin involvement. Taken in connection with Cases III and IV, however, which occurred in the same ward and in patients who were quite closely in contact with Patient II, it seems fairly reasonable to assume that they were all of the same character. Cases of herpes zoster have been extremely rare in this hospital and the occurrence of three cases in the same ward within a very short period of time suggests very strongly a transference of infection from one case to the other. That Case II was not one of herpes simplex also seems fairly certain from the negative results obtained by inoculation of rabbits' eyes with vesicle material.

In making the animal experiments we employed various methods which were suggested largely by the technique used by previous observers, especially by those who have reported results which were considered positive. In making inoculations into the corneas the technique recommended by Lipschütz was employed as far as possible. Young rabbits were used and the material was obtained from fresh vesicles early in the disease and inoculated with as little delay as possible. The material injected into rabbits' eyes was obtained from seven cases and twenty-four rabbits were used. In judging of the results obtained in this kind of experimentation great caution must be observed. Our experience convinces us that slight opacities occurring along the lines of scarification and mild conjunctivitis cannot be held to indicate the effect of a specific virus. As regards the interpretation of the microscopic changes found, we were quite familiar with the appearance of intranuclear inclusion bodies as seen in the lesions of experimental herpes simplex and the filterable virus (Virus III) indigenous to rabbits described by Rivers and Tillett (5). We

also had no difficulty in finding intranuclear inclusions in the sections of skin removed from patients. It is not likely, therefore, that these structures were overlooked in our study of the sections. Briefly stated, although the material studied was satisfactory and in spite of the fact that a considerable number of animals were used for each case, we have been unable to confirm the observations of Lipschütz regarding the experimental production of specific lesions in the corneas of rabbits. We realize that this is only negative evidence and therefore not of conclusive importance in view of Lipschütz's observations. It indicates, however, that the production of specific lesions in rabbits' eyes with material from herpes zoster vesicles is extremely difficult and that successful results may be a matter of chance, depending, possibly, on peculiar susceptibility on the part of the rabbits. In view of the fact, however, that a careful analysis of the positive results reported by other observers shows that the conclusions were based on insufficient evidence, we believe that further work is necessary before the successful inoculation of the rabbits' corneas with herpes zoster virus can be accepted as fully demonstrated. To make the evidence convincing specific lesions should be obtained with a fair degree of regularity and the virus should be successfully transmitted through at least two generations. Apparently the latter was not attempted by Lipschütz.

Intracerebral inoculations into three rabbits with material from two cases (Nos. I and IV) were made. Two rabbits were also inoculated intraspinally with material from one case (No. IV). None of these animals showed any reaction. In the case of one of the animals inoculated into the brain (Case I) although this rabbit showed no symptoms, we thought it conceivable that the susceptibility of the species for the virus might be so slight that no obvious lesion had been produced. Nevertheless it was thought that the virus might possibly remain alive at the seat of inoculation and by repeated transfers become adapted to the rabbit. This phenomenon has been observed by Noguchi with vaccine virus, and by Rivers and Tillett with the rabbit virus isolated by these workers. This possibility was tested by us by making serial corneal and brain inoculations. Corneal transfers were carried through fourteen animals in series, and brain transfers through ten. No specific lesions developed in any of the animals.

The work of Teague and Goodpasture suggested that the skin might be rendered more susceptible to infection by previous treatment with tar. Material from two cases (Nos. I and VIII) was inoculated into the tarred skin of guinea pigs and rabbits. The material was injected intracutaneously and also rubbed into the scarified skin. No reaction was obtained in any of the animals.

Finally, the transmission of herpes zoster to monkeys was attempted. Blanc and Caminopetros, and Bastai and Busacca, as discussed in the review of the literature, inoculated monkeys (*Macacus*) in various ways, without success.

It was thought possible that although monkeys of the genus *Macacus* might be refractory, monkeys of another genus might prove susceptible. Consequently, besides the inoculation of two *Macacus* monkeys, attempts were made to infect five vervets. Moreover, in view of the fact that the virus of vaccinia and the rabbit virus of Rivers and Tillett could be successfully cultivated in the testicle, intratesticular inoculations were employed. The testicles were removed at varying periods following inoculation. Numerous sections of these testicles were made and examined, but in no instance were any lesions found which could be interpreted as specific. No cells containing intranuclear inclusion bodies were found. These experiments, therefore, have also led to purely negative results.

This report of our work is made at the present time because a considerable amount of literature has been published which gives the impression that herpes zoster has been successfully transmitted to animals. Although the observations of Lipschütz are suggestive, it is important that they be confirmed by further investigations.

Until herpes zoster can be regularly transmitted to animals and cross-immunity tests be carried out, the relation of the virus of herpes zoster to that of herpes simplex remains a matter of speculation. In view of the fact that herpes simplex can be easily and regularly transmitted to rabbits, whereas in the hands of a large number of investigators similar experiments with herpes zoster are completely negative, it does not seem likely that the etiological agent concerned in these two diseases can be absolutely identical.

The question of the identity or non-identity of herpes zoster and varicella is even more difficult to answer, because at present neither

of these infections is readily transmissible to animals. The work of Kundratitz is extremely interesting. His observations, aside from indicating a close immunological relationship between herpes zoster and varicella, are important in that they seem to show the presence of a transmissible virus in the vesicles of herpes zoster. The only question that arises is whether the cases of herpes zoster from which Kundratitz was able to make successful transfers were true cases of idiopathic herpes zoster.

<div align="center">CONCLUSION.</div>

Attempts to inoculate rabbits, guinea pigs, and monkeys with material obtained from nine cases of herpes zoster have proved unsuccessful.

<div align="center">BIBLIOGRAPHY.</div>

1. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673.
2. Tyzzer, E. E., *Philippine J. Sc.*, 1906, i, 349.
3. Lipschütz, B., *Arch. Dermat. u. Syph., Orig.*, 1921, cxxxvi, 428.
4. Goodpasture, E. W., *Am. J. Path.*, 1925, i, 1. Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923–24, xliv, 121, 139.
5. Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673; 1924, xxxix, 777; 1924, xl, 281.
6. Grüter, W., *Ber. 42. Versamml. ophth. Ges.*, 1920, 162; *Klin. Monatsbl. Augenheilk.*, 1920, lxv, 398.
7. Löwenstein, A., *Münch. med. Woch.*, 1919, lxvi, 769.
8. Löwenstein, A., *Klin. Monatsbl. Augenheilk.*, 1920, lxiv, 15.
9. Doerr, R., and Vöchting, K., *Rev. gén. ophth.*, 1920, xxxiv, 409.
10. Kling, C. A., *Berl. klin. Woch.*, 1913, 1, 2083; 1915, lii, 13.
11. Lipschütz, B., *Wien. klin. Woch.*, 1924, xxxvii, 183.
12. Marinesco, M. G., *Bull. Acad. méd.*, 1922, lxxxviii, series 3, 487; *Riforma med.*, 1922, xxxviii, 1211.
13. Marinesco, M. G., and Draganesco, S., *Rev. neurol.*, 1923, xxx, 30.
14. Truffi, M., *Pathologica*, 1922, xiv, 565.
15. Mariani, G., *Policlinico, sez. prat.*, Rome, 1922, xxix, 1193; *Gior. ital. mal. ven.*, 1922, lxiii, 1199.
16. Blanc, G., and Caminopetros, J., *Bull. Soc. franç. dermat. et syph.*, 1922, xxix, 294.
17. Meineri, P. A., *Pathologica*, 1922, xiv, 772.
18. Kraupa, E., *Münch. med. Woch.*, 1920, lxvii, 1236.
19. Baum, O., *Dermat. Woch.*, 1920, lxx, 105.
20. Teissier, P., Gastinel, P., and Reilly, J., *Compt. rend. Soc. biol.*, 1922, lxxxvi, 73.

21. Kooy, J. M., *Klin. Monatsbl. Augenheilk.*, 1921, lxvi, 75.
22. Netter, A., and Urbain, A., *Compt. rend. Soc. biol.*, 1924, xc, 461.
23. Bloch, M., and Terris, E., *Compt. rend. Soc. biol.*, 1924, xc, 1394.
24. Simon, C. E., and Scott, J. M., *Am. J. Hyg.*, 1924, iv, 675.
25. Doerr, R., *Zentr. Haut- u. Geschlechtskrankh.*, 1925, xvi, 481.
26. Luger, A., and Lauda, E., *Z. Hyg. u. Infectionskrankh.*, 1921, xciv, 206;
    Centr. Bakt., *1. Abt.*, *Orig.*, 1923–24, xci, 205; *Klin. Woch.*, 1925, iv, 209.
27. Grüter, W., *Münch. med. Woch.*, 1924, lxxi, 1058.
28. Bastai, P., and Busacca, A., *Schweiz. Arch. Neurol. u. Psychiat.*, 1925, xvi, 56.
29. Cipolla, G., *Ann. clin. med. e med. sper.*, 1923–24, xiii, 175.
30. Teague, O., and Goodpasture, E. W., *J. Am. Med. Assn.*, 1923, lxxxi, 377;
    J. Med. Research, 1923–24, xliv, 185.
31. Lipschütz, B., *Centr. Bakt.*, *1. Abt.*, *Orig.*, 1924, xciii, 361.
32. Kundratitz, K., *Monatschr. Kinderheilk.*, 1925, xxix, 516. Lipschütz, B.,
    and Kundratitz, K., *Wien. klin. Woch.*, 1925, xxxviii, 499. Kundratitz, K.,
    Z. Kinderheilk., 1925, xxxix, 379.
33. von Bókay, J., *Jahrb. Kinderheilk.*, 1924, cv, 8.
34. Doerr, R., *Zentr. Haut- u. Geschlechtskrankh.*, 1924, xiii. 417; 1924, xv, 1, 129,
    289.

BACTERIOPHAGE TESTS ON THE MECONIUM OF ABORTED FETUSES.

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Since d'Hérelle's[1] advocacy of the living nature of the bacteriophage there has been a divergence of opinion as to its real nature and origin, although its widespread prevalence in the intestinal tract of adult man and animals meets with general acceptance. The stimulus for the present work lay in the hopes that it might add further information to the theory that bacteriophagous lysis can be initiated by tissue cells.

Bordet and Ciuca[2] were among the first to question the living nature of the bacteriophage, for by injecting *Bacillus coli* intraperitoneally into guinea pigs they demonstrated that the resulting exudate could bring about transmissible lysis of the injected strain. The source of the lytic substance was attributed to the leucocytes.

Wollstein[3] and Kuttner[4] have repeated these tests with some success, although it appears that this method is not without its difficulties. Human peritoneal exudates examined by Wollstein contained a *coli* phage in a case of peritonitis and a dysentery phage in a case of fatal dysentery. Kuttner reports the extraction of phage from the intestinal mucosa and liver cells of normal guinea pigs, as well as demonstrating its presence in normal rabbit serum. Callow[5] isolated phage from the lesions in sixteen cases of staphylococcus infection, and McKinley[6] from seventeen different sources in normal and pathological tissues of man and animals.

[1] d'Hérelle, F., authorized translation by Smith, G. H., The bacteriophage. Its rôle in immunity, Baltimore, 1922.

[2] Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1293.

[3] Wollstein, M., *J. Exp. Med.*, 1921, xxxiv, 467.

[4] Kuttner, A. G., *J. Bact.*, 1923, viii, 49.

[5] Callow, B. R., *J. Infect. Dis.*, 1922, xxx, 643.

[6] McKinley, E. B., *J. Lab. and Clin. Med.*, 1923–24, ix, 185.

The impression to be gathered from the interpretations of these investigators is that the tissue cells may play a rôle in the production of bacteriophage. Objections to the leucocyte theory of Bordet and Ciuca were early made by d'Hérelle on the basis of his previous experiments indicating that the bacteriophage could penetrate the intestinal mucosa and migrate by way of the blood stream throughout the body. Its presence in peritoneal exudates he holds is in accordance with this phenomenon. All experiments having to do with tissue cells as a source of bacteriophage are subject to a like interpretation in the presence of an intestinal tract with complex bacterial and associated bacteriophagous flora.

The study of cattle abortion as carried on in this laboratory appeared to offer material admirably adapted for the elimination of the above objection, since the flora of the rectal contents of aborted fetuses was usually reduced to its simplest terms; namely, *Bacillus abortus*.

Six fetuses ranging in age from 6 months to approximately full term were tested. Any which had breathed and from which an organism other than *B. abortus* was recovered from the rectal contents were discarded. The organism was not isolated from one of the included cases, although as it was recovered from the uterine discharges of the dam it was undoubtedly present. 16 to 20 gm. of meconium were removed aseptically to 30 to 100 cc. of sterile fermented bouillon, shaken well, centrifugalized, passed through a Berkefeld filter, and then placed in the ice box until used. The filtrates diluted 1:10 in slightly alkaline bouillon were tested against twelve strains of *B. abortus* isolated in this laboratory, of which three were derived from the above fetuses. One of the latter failed to grow in bouillon, as did one of the other strains. Inoculum into the diluted phage was 0.5 cc. of a 3 day old bouillon culture. After a 24 hour incubation at 37°C., 0.5 cc. quantities were inoculated onto the surface of slightly alkaline agar slants. Control tubes without the addition of filtrate were used for each culture. All tubes were examined up to a week for evidence of lysis. In no instance, however, was there any trace of lysis to be observed.

In view of these frankly negative results, it was decided to test the filtrates against bacteria other than *Bacillus abortus*. For this purpose a number of different strains of colon bacilli isolated in this laboratory from various calf conditions were used,—34 in all, although no filtrate was subjected to the whole list. A lysable strain of human origin was also included.

In these tests the filtrates were diluted 1:10 with alkaline bouillon and inoculated with one loopful of an emulsion made from an 18 hour culture grown on slanted agar. Immediately following inoculation, 0.5 cc. quantities were spread over the surface of agar slants. Culture controls without filtrate were included. Incubation was at 37°C. At no time up to 6 days was there any evidence of lysis, although the B. coli human strain in the presence of its homologous bacteriophage was completely lysed. Sterility tests on the filtrates yielded no growths.

DISCUSSION.

In so far as these tests on meconium show, there is no indication that the fetuses produced a bacteriophage in the presence of a specific infection, or that one was naturally present for numerous colon strains of bovine origin. That fetal tissues are capable in other respects of reacting against *Bacillus abortus* has been indicated recently by Theobald Smith[7] in studies on fetal pneumonias.

In association with other work the writer has been able to demonstrate in the rectal contents of young calves a bacteriophage active for bovine strains of colon bacilli as well as for the human type used above.[8]

SUMMARY.

The theory that tissue cells are the source of bacteriophage was objected to by d'Hérelle on the grounds that bacteriophage in the intestinal contents could penetrate the intestinal mucosa and migrate throughout the body and that any demonstration of its presence within the body fluids was in accordance with this phenomenon. The present work sought to overcome this objection by using tissues which had been exposed to but a single organism,—*Bacillus abortus*. Filtrates of meconium from six aborted bovine fetuses were tested against several strains of *Bacillus abortus*, but no evidence of a lytic principle could be demonstrated. Neither could it be shown that they contained a substance which would initiate lysis when tested against numerous strains of bovine colon bacilli.

[7] Smith, T., *J. Exp. Med.*, 1925, xli, 639.

[8] Since this work was completed, the writer's attention has been called to a paper by Surányi, L., and Kramár, E., *Monatschr. Kinderheilk.*, 1924, xxviii, 330. In a study of ten new-born infants no bacteriophage active for organisms of the colon-dysentery group could be demonstrated in the meconium, and, furthermore, such a bacteriophage was not detected until after the 14th day of life.

SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS.

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PLATES 26 TO 28.

Various bovine infections characterized by a pyelonephritis have been recognized in Europe.

Guillebeau[1] described a condition of such nature in 1888, and several have since reported diseases affecting the pelvis of the kidney. We would infer from the literature that renal diseases among cattle are widespread in Continental Europe. A number of etiological agents have been recognized. Höflich[2] and Enderlen[3] both cultivated from the affected kidneys of a few cases a Gram-positive diphtheroid which Enderlen names *B. pyelonephritis boum.* Ernst,[4] who studied the kidney lesions from a series of cases, encountered a similar organism in a certain number; while from others he obtained *B. pyogenes* and *B. coli.* Owing to the difficulty encountered in reproducing the disease in cows with organisms similar to those isolated by Höflich and Enderlen, considerable confusion seems to have arisen. Ernst, from certain experimental observations, even questions the etiological relationship of the diphtheroid to the processes encountered. Joest,[5] however, considers pyelonephritis a specific infectious disease of cattle and calls attention to the fact that there is no disease having a similar etiology and pathology.

We are not aware that diseases of this nature have been reported in this country.

The Appearance of Pyelonephritis in New Jersey.

In October, 1924, our attention was called to a cow affected with nephritis. At the owner's request the cow was autopsied. Lesions of

[1] Guillebeau, *Schweiz. Arch. Tierheilk.*, 1890, xxxii, 224.
[2] Höflich, D., *Monatsh. prakt. Tierheilk.*, 1891, ii, 337.
[3] Enderlen, D., *Z. Tiermed.*, 1890-91, xvii, 325.
[4] Ernst, W., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 549.
[5] Joest, E., Spezielle pathologische Anatomie der Haustiere, Berlin, 1924, iii.

pyelonephritis were noted, and a diphtheroid was isolated from the kidney. A more detailed report of the pathological findings will be given later. It was possible to obtain a relatively complete history of importations into this herd during the past few years, and in addition, a record had been kept of the deaths and the autopsy findings. In November, 1922, a number of cows were purchased in the Middle West. The first loss from nephritis occurred in April, 1923. Another cow died in May, 1923. During 1924 two cows died of the disease, one in July and the other in August. We autopsied a severe case (No. 1132) on October 27, 1924. Since then other cases have been recognized.

A larger herd in which the practice is to introduce a large number of cows each year has been under observation by various members of the staff for a considerable period. Infections of the kidney were known to exist; in two instances[6] in which bacteriological examinations had been made *Bacillus pyogenes* was obtained. Inasmuch as there had been several importations during 1922 from the same district as that referred to in the smaller herd, search was instituted for such infections in the larger herd.[7] On November 9, 1924, a cow (No. 1138) was found dead. Bacteriological examination of the kidneys revealed an organism similar to that obtained from the case mentioned. Between November, 1924, and May, 1925, twenty such infections have been recognized. Since certain cases illustrate points to be considered further on the protocols of a number will be given.

No. 1132.—A Holstein cow, one of the smaller herd referred to, had been purchased in the Middle West in 1922. It had given birth to a calf in August, 1923. On Oct. 20, 1924, the milk flow suddenly lessened. On Oct. 27, the cow was examined. Unthriftiness was noticed. The temperature was 102°. The appetite was good and the feces normal. Weakness was pronounced. The pulse and respiratory rate were normal. A little turbid urine was obtained. The cow was slaughtered and autopsied at once.

The lesions were confined to the urinary apparatus. Both kidneys were enlarged. The cortex of the right kidney was clay color. The consistency was firm and tough. Scattered over the surface were many irregular, slightly raised, gray patches. They varied in size from barely visible points to patches which occupied

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[6] We are indebted to Dr. Theobald Smith for these data.

[7] Since the data have been gathered together the disease has been encountered in a third herd.

practically a whole lobe. The capsule was adherent over such areas. On section the processes frequently extended through the cortex and into the medulla. The medulla was bright red except in the portions where the gray patches had extended from the cortex. The pelvic space was dilated and filled with a greenish yellow purulent exudate. The left kidney presented similar lesions. Both ureters were increased in diameter. The left measured 2 cm. in diameter. The walls were thickened and the mucosa congested. An exudate similar to that observed in the kidney pelvis was present in the ureters. The bladder was contracted. It contained 200 cc. of turbid urine. The walls were extremely thick. The mucous membrane was partially covered with an adherent, rough, grayish white exudate. The urethra presented no gross lesions. The uterus was apparently normal. A specimen of urine obtained from the bladder contained considerable yellowish white stringy material. Examination showed the following: Reaction, alkaline; specific gravity, 1.016; albumen, present (Esbach's, 0.4 per cent); sugar, not present. Leucocytes and bacilli in enormous numbers were present in the sediment.

Inoculations into various media were made from the heart blood, spleen, and kidneys. The tubes inoculated with heart blood remained sterile. Bits of both kidneys implanted in the condensation water of blood and plain agar slants developed characteristic diphtheroids both in pure culture and mixed with streptococci. In all instances the diphtheroids were present in large numbers. One tube inoculated with a fragment of spleen developed the same organism in pure culture. Films and fresh preparations from the urine and the exudate from the pelvis of the kidney revealed, on microscopic examination, the diphtheroids in large masses.

Fixed and stained sections from the spleen and uterus revealed no abnormalities on microscopic examination. Many of the liver cells were granular; some were infiltrated with fat. The sections from the kidneys presented severe lesions. The cortex had been invaded by densely packed masses of round cells. In places considerable of the cortical structure had been practically obliterated by the infiltrative process. Leucocytes in dense masses were frequently observed throughout the cortex. Deep within the cortex connective tissue hyperplasia had occurred. Leucocytes in large numbers had invaded the connective tissue. The adjacent tubules were compressed and their epithelium degenerated. In certain portions of the medulla considerable interstitial hyperplasia existed. The collecting tubules were compressed and frequently filled with epithelial casts. In other portions the process was more acute. The blood vessels were engorged and the tubular epithelium degenerated. Often the tubules were plugged with polymorphonuclear leucocytes and fibrin. The interstitial tissue was edematous.

Much of the mucosa of the ureters was necrotic or degenerated. The free surface frequently consisted of a mass of granular exudate containing leucocytes, red cells, and necrotic epithelium. In portions where the process was not as advanced many of the epithelial cells were degenerated and leucocytes in large numbers had invaded the mucous layer. The submucous connective tissue was

edematous and the capillaries engorged with red cells. The edema extended into the muscular layers.

No. 1138.—Purchased in the Middle West Aug. 7, 1922. Sept. 29. Calved normally. Jan. 29, 1924. Bred. Aborted Sept. 23. Died Nov. 9. On Nov. 10, both kidneys, the bladder, and the uterus were submitted for examination. There was considerable decomposition.

The right kidney was enlarged. It measured 25 × 15 × 5 cm. The color was yellowish gray. The consistence varied; in places it was firm, in others softened. On the whole both the cortex and medulla appeared narrower than normal. The line of demarcation between the cortex and medulla was indistinct. The more superficial portions of the medulla were bright red. Deeper the medullary structures appeared necrotic. The pelvis was greatly dilated and partially filled with necrotic material, and blood-stained urine.

The left kidney was also enlarged. It measured 23 × 13 × 6 cm. The color was yellowish red. The consistency was tough. On section the color varied from yellowish pink in the cortex to deep red in the medulla. The papillæ were necrotic. The pelvis was dilated and filled with blood-stained urine and foul smelling exudate.

Only small portions of the ureters were obtained. They were greatly dilated. The walls were appreciably thickened. Granular tufts were adherent to the mucous membrane. The tubes were partially occluded with yellow exudate.

The bladder was contracted. It contained 300 cc. of red-stained urine. The mucous surface was reddened and swollen; patches of grayish white exudate adhered to the mucosa. The urine obtained from the bladder was turbid and of a deep red color. The specific gravity was 1.020. The reactions for albumen were strong (Esbach's, 1.5 per cent). The urinary sediment contained red cells, leucocytes, cocci, and diphtheroids in large numbers.

There was marked decomposition of the uterus. The walls were moderately thickened. The mucosa was necrotic and covered with a foul smelling, greenish white exudate.

Cultures taken from the kidney cortex showed only the presence of anaerobic putrefactive types. Those from the kidney pelvis and urine developed cocci in small numbers and diphtheroids in enormous numbers. The uterus contained an enormous number of bacteria. *B. pyogenes* predominated but diphtheroids similar to those found in the urine and kidneys were also found in limited numbers.

Inasmuch as the material was not fixed in Zenker's until decomposition had taken place the sections were not particularly illuminating. The kidney cortex contained well defined wedge-shaped areas infiltrated with round cells. The glomerular epithelium was degenerated and congested. The tubular epithelium in certain portions appeared as granular, pink-staining cells without nuclei. On the whole the connective tissue proliferation was more marked in the medulla. Pressure changes were marked. The epithelium lining the bladder and ureters consisted of a narrow band of degenerated cells. The connective tissue beneath was edematous. It had proliferated. Leucocytes and round cells had invaded

the submucous structures. Edema extended throughout the muscular coats. Much of the epithelium of the uterus was necrotic. Leucocytes and round cells were present in large numbers in the submucous structures.

The following cases are more fully illustrative of the condition, since it was possible to diagnose them during life and to verify our clinical findings at autopsy.

No. 1143.—This cow was purchased in the Middle West in December, 1921; bred in November, 1923, and gave birth to a normal calf Aug. 28, 1924. On Nov. 11, about 500 cc. of turbid urine was passed. The later portion of the urine was dark red in color. After considerable straining 10 or 15 cc. of bright blood was evacuated. The blood was not included in the sample collected. The urine was alkaline; specific gravity 1.037; responded strongly to HNO₃, and gave an Esbach reading of 0.25 per cent. There was considerable sediment. It consisted of red cells, leucocytes, and masses of diphtheroid bacilli. Plates prepared from the sediment developed the diphtheroids in overwhelming proportions. On Nov. 12 a blood culture was made. It remained sterile. A physical examination on that day revealed little of note. The temperature was 38.8°C.; pulse 66; respiration 20. No abnormalities were noted in the heart action. The lungs appeared normal. The digestive system appeared to be functioning properly. Rectal examination failed to reveal enlargement of the kidneys. The cow was an extremely high type of dairy animal, the milk secretion averaging about 20 quarts daily. The urine was again examined on Nov. 21. The findings were similar to those recorded except that more albumen was present. On Nov. 26 the cow was slaughtered at an abattoir.

The lesions were confined to the urinary tract. The kidneys appeared normal. The capsule was not adherent. There were no accumulations of urine or other material in the pelvis. The ureters traced from the kidneys to the bladder failed to reveal abnormalities. The bladder appeared as a shrunken, wrinkled, thick walled sac containing only 25 to 30 cc. of blood-stained urine. It measured 12 cm. long by 10.5 cm. wide. The serous surface was congested. The walls were 1.5 cm. thick. The mucosa was swollen. It varied in color from bright yellow to scarlet. Scattered over the ventral surface in the region of the urethra were a few tiny, raised, grayish white patches of exudate. Blood clots partially adherent to the mucosa were also observed. The urethral mucosa appeared normal. The genital tract appeared normal.

The tubes inoculated with bits of kidney cortex and medulla remained sterile. The urine in the bladder when plated contained only diphtheroids. From both horns of the uterus diphtheroids were obtained in small numbers.

Microscopic examination of fixed and stained sections from the kidneys, ureters, uterus, and urethra revealed little abnormal. The lesions in sections from various portions of the bladder varied considerably. Certain areas showed relatively little degeneration of the epithelium. The nuclei of occasional cells were pycnotic.

Leucocytes and round cells had made their way into the mucous coat in small numbers. The submucous coats were edematous and the capillaries engorged. In other portions the mucous membrane was more severely affected. The cells were degenerated or necrotic. Masses of leucocytes were observed in the mucous layer. In circumscribed areas remnants of the epithelial cells were still discernible in dense masses of necrotic red cells, leucocytes, and fibrin. The structures beneath were also invaded by leucocytes. Proliferation of the underlying connective tissue was noted. Bacilli in great numbers were observed in the necrotic material. They were not seen in the deeper structures.

No. 1152.—Grade Holstein cow, purchased in April, 1923. Two normal parturitions, one in June, 1923, and the other in June, 1924.

Jan. 6, 1925. 900 cc. of turbid, deep red urine obtained. Alkaline in reaction; specific gravity 1.025. Tests for albumen were positive (Esbach's, 0.5 per cent). Sugar was not present. On centrifugation, considerable sediment was observed. It was composed of red cells, leucocytes, masses of diphtheroids, and a few cocci. Plate cultures prepared from the sediment developed over 95 per cent colonies of diphtheroids.

Jan. 12. Blood culture negative.

The animal was killed on Jan. 16. The lesions were confined to the urinary tract. The right kidney appeared normal except that one lobule was grayish white in color and tougher than the others. On section the gray color extended through the cortex and into the medulla in the form of fine interlacing lines. One lobe of the left kidney protruded sharply above the others. On palpation it fluctuated. It was composed of a thin wall of cortical tissue, 2 to 3 mm. in thickness, which enclosed clear urine. Both ureters failed to show gross changes throughout their entire length. The bladder was contracted (Fig. 1). The walls were thick. The mucous membrane was swollen and varied from pink to dark red in color, that of the ventral portion was deeply reddened. Patches of variable size of dirty grayish white exudate covered this portion of the mucosa. The exudate when pulled from the mucosa left a raw, reddish gray surface. The mucosa in the more dorsal portions was thrown up into livid, deep or bright red, glistening folds. The urethra presented no abnormalities. The normal uterus contained a fetus.

Stained sections of the kidneys on microscopic examination failed to show abnormalities except for a few small accumulations of round cells. The ureters were normal. The bladder epithelium (Fig. 3) was necrotic throughout two sections. Much of it had disappeared. Small portions were still visible as thin bands of necrotic cells loosely adherent to the underlying structures. The submucous connective tissue was infiltrated with a serous exudate. The connective tissue filaments were forced far apart; their nuclei appeared as tiny round cells situated at wide intervals. The capillaries were packed with red cells. Hemorrhages extending from the capillaries to the bladder surface were not uncommon. In several instances red cells were being discharged into the bladder space. The urethral epithelium was intact. The submucosa was heavily infiltrated with

round cells. The round cell infiltration extended into the muscular structures. The uterus was normal.

Cultures from the cortex of the left kidney remained sterile. Those from the urine within the cyst developed cocci and diphtheroids.

No. 1153.—A grade Holstein cow, purchased Aug. 25, 1924. Calved normally Sept. 5. On Jan. 8, 1925, it was noted that the cow passed turbid urine and at the close of urination bright blood. A little material obtained by swabbing the mucosa about the urethral opening revealed on examination the characteristic bacilli. On Jan. 9, 200 cc. of dark red turbid urine containing blood clots was procured. The reaction was alkaline; specific gravity 1.028; albumen reaction strong, 1.0 per cent; tests for sugar were negative. After centrifugation there was considerable sediment consisting of red cells, blood clots, leucocytes, epithelial cells, and diphtheroids in enormous numbers. Plate cultures developed diphtheroids in practically pure culture. On Jan. 12, blood was obtained for culture. It remained sterile.

On Jan. 16, the cow was slaughtered. The left kidney presented no abnormalities. One lobe of the right kidney was sharply protruded. It was a mottled reddish gray in color, tough in consistency. A calculus was found occluding a large tubule at the tip of a papilla. The pelvic space was increased in size and contained a little yellow exudate. The right ureter appeared normal; the diameter of the left was increased. The bladder (Fig. 2) was contracted and contained 500 cc. of turbid urine. The bladder walls appeared thick. The mucous membrane was swollen and varied in color from yellow to deep red. Patches of grayish white exudate were adherent to the mucosa. They varied in size and general outline. One well isolated round area measured 1.7 cm. in diameter. Its borders were slightly raised above the surrounding epithelium but its center was depressed. In places isolated patches of exudate had become more or less confluent. Attempts to forcibly separate the exudate from the underlying structure left a rough, reddened surface.

Examination of the stained sections of tissues fixed in Zenker's fluid revealed little abnormal in the left kidney. Sections of the mottled lobule of the right kidney revealed a number of wedge-shaped areas rich in round cells. The base of the wedge was located at the surface of the cortex and extended downward a variable distance. A finger-like process of connective tissue, which extended from the capsule between two lobules, could be traced to the thick walled cyst which originally contained the calcareous body. In the papillæ a considerable proliferation of interstitial tissue had occurred. The left ureter failed to show microscopic changes. The right ureter was about twice as thick as the left; the mucous membrane was intact. Small accumulations of round cells were noted just beneath the mucosa. The submucosa was edematous and the capillaries moderately engorged. The bladder lesions were severe. The cells of a considerable portion of the mucous membrane were degenerated or necrotic. Considerable patches of the epithelium had exfoliated so that the bladder surface appeared as a pink-staining, granular mass containing a few leucocytes. Patches of fibrin-

ous exudate, which contained only a few cells, partially covered the underlying structure. The vessels of the submucosa were dilated with red cells and fibrin. The connective tissue of the submucosa had proliferated. It was markedly edematous. The edema extended into the muscular coats. In many of the fixed and stained sections of the bladder it was difficult to find the organism. Apparently it is not highly invasive, since it may readily be found in the urine but has never been encountered in the deeper structures. In a few instances sections of the bladder showing the superficial distribution of the organisms have been obtained. Figs. 4 and 5 are illustrative. Fig. 4 reveals under a low magnification dense masses of bacilli lying in the exudate. Fig. 5 is a higher magnification of the edge of one of the black masses. The characteristic bacilli are plainly visible. The uterus was normal.

Cultures from the cortex of the left kidney and uterus remained sterile.

No. 1154.—A grade Guernsey purchased in the Middle West in 1922; passed through a normal parturition in August, 1923, and in August, 1924. Early in November, 1924, gastrointestinal disturbances were noted. On Nov. 10, the cow attracted attention because of its general poor condition. Urine was first obtained on Nov. 11. It failed to react to tests for albumen. On Dec. 3, 250 cc. of turbid urine was obtained. Its reaction was alkaline; specific gravity 1.037· albumen strong, 1.4 per cent. There were leucocytes, red cells, and diphtheroids in enormous numbers in the sediment (Fig. 6). The bacilli were readily cultivated. On Dec. 27, a blood culture was negative. Examination of the urine on three other occasions gave about the same results. On Jan. 16, 1925, the cow was slaughtered.

The right kidney and ureter were apparently normal. The left kidney (Fig. 7) was mottled grayish pink. Certain lobules were entirely gray, while others contained more or less distinct, irregular gray patches. The gray color in certain lobules extended throughout the cortex and into the medulla. The line of demarcation between the cortex and medulla was indistinct. In two lobules the cortex and medulla were involved to about the same extent. The kidney was tough. The pelvic space was diminished and infiltrated with connective tissue. The walls of the large collecting tubules were greatly thickened. The mucosa of the ureter appeared normal but the walls were thickened. The bladder was contracted and contained only a little blood-tinged urine and several blood clots. The bladder walls were thickened. The swollen mucous membrane was bright red in color and covered with non-adherent purulent mucus.

The stained sections of the right kidney revealed little abnormal except periglomerular and interstitial accumulations of round cells. Those of the right ureter appeared normal. The lesions in the left kidney (Fig. 8) were pronounced. The capsule was thickened and infiltrated with round cells. In places much of the normal cortical structure had disappeared. Occasionally it was possible to recognize the remains of a glomerulus or a tubule. In the main the process consisted of a connective tissue proliferation rich in both small and large round cells. A few of the remaining tubules were filled with leucocytes. The process was less

marked in the medulla, but here there existed irregular accumulations of round cells in areas of proliferating connective tissue. The epithelium of many of the collecting tubules was degenerated and the lumen filled with polymorphonuclear leucocytes. Deep within the medulla the number of tubules was decreased, and the intertubular connective tissue had proliferated and produced marked pressure changes. The walls of the left ureter were thickened, due to edema of the submucous connective tissue. Round cell accumulations were noted in the submucosa. The mucous membrane was intact. The bladder mucosa was more or less degenerated. In places all that remained were a few necrotic epithelial cells sparsely sprinkled over the submucous structures. There was marked edema of the submucous connective tissue. The capillaries were greatly dilated and filled with red cells. Accumulations of round cells were frequent throughout this area. The edema extended into the muscular coats. The mucosa of the urethra was intact but great masses of round cells were present beneath the mucous membrane. Microscopic lesions were not encountered in the sections of the uterus.

Bits of the cortex from both kidneys when implanted into the condensation water of plain agar and blood agar slants remained sterile. Media inoculated from the uterus remained sterile.

No. 1174.—On Jan. 7, 1925, the urine failed to react to tests for albumen and diphtheroids could not be detected in the sediment. On Jan. 23, about 30 cc. of bright blood was passed. This when cultured revealed enormous numbers of diphtheroids. On Jan. 28, turbid, blood-stained urine was obtained. It responded to the albumen tests, 0.5 per cent by Esbach's method. Examination of the sediment revealed fibrin, red cells, leucocytes, and diphtheroids. Urine voided on Jan. 29 was similar in character. Its specific gravity was 1.036. From this time until Feb. 10 the urine was examined at daily intervals. Blood and the bacilli were always present in the sediment. On Feb. 18 the cow was killed.

The right kidney and ureter failed to show abnormalities. In the left kidney one of the large collecting tubules was dilated. It had the appearance of a thick walled cyst filled with clear urine. The left ureter was apparently uninvolved.

The bladder was contracted. The mucous membrane was swollen, bright red in color, and thrown up into broad folds. Several small irregular blood clots were adherent to the mucosa. In other places the membrane was covered with a thick, non-adherent layer of mucopurulent exudate. The urethra was apparently normal. The vagina and uterus presented no gross changes.

Summary of Clinical and Pathological Findings.

From the protocols submitted it will be noted that the cows presented no marked symptoms. The temperature was always within normal limits. Some of the cows were thin but others were in good flesh. A considerable number were admirable dairy types and continued to produce a large quantity of milk until slaughtered.

TABLE I.

The Lesions Encountered in the Urinary Tract of the Thirteen Cases.

No.	Termination.	Bladder.	Ureters.	Kidneys.
1132	Advanced case. Killed.	Severely involved.	Both thickened.	Both severely involved.
1138	" Died.	"	" "	" " "
1143	Slaughtered for food.	"	Not involved.	Not involved.
1151	" "	"	" "	Right: calculous; fibrosis of one lobe.
1152	" "	"	" "	" fibrosis of several lobes. Left: one lobe cystic.
1153	" "	"	Right: thickened. Left: not involved.	Right: calculous; fibrosis of one lobe. Overgrowth of connective tissue in pelvic space. Left: apparently normal.
1154	" "	"	Right: " " Left: thickened.	Right: not involved. Left: severely involved.
1172	" "	"	Not involved.	Cortex of both sprinkled with tiny round cell areas. No lesions of pelvis.
1173	" "	"	" "	Not involved.
1174	" "	"	" "	" "
1193	" "	"	Both thickened.	" "
1194	" "	"	Right: " Left: not involved.	Right: severely involved. Left: fibrosis of several lobes.
1204	" "	"	Not obtained.	Pelvis of both invaded by the bacilli.

The most noticeable symptom is the condition of the urine. Attention is frequently drawn to the frequency with which urine is voided. As a rule in the early cases the urine is turbid and the sediment on microscopic examination reveals red cells and leucocytes. Later it is blood-stained and frequently contains blood clots. Usually the cows strain considerably after urination and are apt to pass bright red blood in small quantities.

The lesions may be confined to the bladder, or, in addition, one or both kidneys may be involved. Thickening of the ureter leading from the affected kidney may be pronounced. The bladder is usually contracted. The walls are from 1 to 3 cm. thick. The mucous membrane is edematous and thrown into broad folds. Its color varies from pink to scarlet. In certain cases the mucous layer is covered or replaced by a grayish white, adherent exudate. The affected kidney is enlarged and often firm. It is mottled from gray to red. The pelvic space may be contracted and filled with a thick yellow mass of exudate. The walls of the larger collecting tubules are greatly thickened.

The bacilli are always present in the urinary sediment in large numbers. Suitable preparations from the pelvis of an involved kidney reveal them in equally large numbers.

We have been able to autopsy 13 cases in all. A brief outline of our findings is appended in the table.

It will be noted from the table that our cases may be divided into several groups. The first comprises Nos. 1132, 1138, 1154, and 1194, cows with one or both kidneys severely involved. All revealed a thickening of the walls of one or both ureters. Nos. 1151, 1152, and 1172 all suffered from severe bladder involvement; the kidney lesion was confined to fibrosis of one or more lobules. Whether the fibrosis results from infection with the diphtheroids is not known. It is of interest to note that similar lesions have been encountered by Theobald Smith[8] in young calves following interference with the normal intake of colostrum. It is possible that this lesion had persisted since early life. The ureters failed to show abnormalities. Cystitis was pronounced in Nos. 1153 and 1193, and in both instances the

[8] Smith, T., *J. Exp. Med.*, 1925, xli, 413.

ureters were thickened.   The lesions were confined to the bladder in
Nos. 1143, 1173, and 1174.

In two instances recovery has been observed.   A single case will
be cited.

Cow 1203 was known to be infected in November, 1923.   A urine examination
in January, 1925, confirmed our diagnosis.   The animal was slaughtered because
of an udder injury in May, 1925.   The bladder although contracted presented no
abnormality except a considerable increase of submucous connective tissue.   The
urine was clear and failed to react to tests for albumen.   The bacilli could not be
cultivated from the sediment.

DISCUSSION.

Among earlier writers considerable discussion has arisen as to
whether the infection is an ascending one or whether the organism
gains entrance to the kidney from elsewhere within the body.

Certain experimental findings have led several, among them Kitt[9] and Ender-
len,[8] to conclude that the disease is a hematogenous infection.   Kitt based his
assumption that the kidney infection originated by way of the blood stream on the
distribution of a miliary type of lesion in both kidneys.   He was further impressed
by an experiment performed by Enderlen who injected the bacilli into the bladders
of rabbits and guinea pigs and failed to note cystitis or nephritis.   Intravenous
injection of culture into normal rabbits also failed to produce lesions.   If, how-
ever, a ureter was ligated and the organisms injected into the blood stream, disease
resulted in the kidney.   Ernst[4] also regards the disease as of hematogenous
origin.   His material was drawn largely from the abattoirs.   In only a few
instances were the bladders examined.   Of 24 cases examined by him he obtained
the tubercle bacillus from 2, $B.$ coli from 8, $B.$ pyogenes from 12, and diphtheroids
from 11.   From his study he concluded that pyelonephritis in cattle is largely a
hematogenous infection, although in certain cases in which the bladder was first
involved the process was regarded as an ascending one.   He further pointed out
that in vaginitis and metritis following parturition the septic material may gain
access to the blood stream and produce a focus within the kidney.   The hematog-
enous origin has been questioned by many, among them Guillebeau and Hess[10]
and Ritzenthaler.[11]   Ritzenthaler, in an analysis of his series of cases, suggested
that the organisms gained access to the bladder by way of the urethra and from
there by the process of growth up the ureters, and finally involved the kidneys.

[9] Kitt, Th., *Path. Anat. Haustiere*, 1901, ii, 471.
[10] Guillebeau and Hess, cited by Ritzenthaler.
[11] Ritzenthaler, M., *J. Comp. Path. and Therap.*, 1910, xxiii, 33.

In the main it seems to us that the several workers have discussed different types of infection and as consequence a generalization or conclusion to cover essentially different processes has resulted. It is also true that in many instances the bladders have not been obtained or that bladder lesions have been overlooked. In kidney disease resulting shortly after parturition, particularly in cases where there has been metritis, the organisms may have gained access to the kidney from the blood stream. The lesion would be largely of the cortex and purulent in character. The organism usually present in bovine metritis, *Bacillus pyogenes*, would be encountered in the kidney. The bladder lesion would be secondary. By contrast, the disease which we have described is relatively specific. We have shown that the kidneys were involved only in certain cases. In a limited number both kidneys were involved, but we have observed involvement in a single kidney in two instances. Evidence exists in certain cases that the pelvis is the first portion of the kidney invaded by the organisms. Here they are found in large numbers, whereas visibly involved portions of the cortex are often sterile when cultured. Involvement of the kidney was always associated with a thickening of the corresponding ureter. In one instance the walls of both ureters were appreciably thickened but the kidneys failed to show lesions. The bladder changes are most characteristic of the disease and occur in all cases. We infer from this evidence that the disease is an ascending infection, the organisms gaining entrance to the bladder and by a process of growth finally reaching the pelvis of the kidney. The organism is not one usually associated with vaginal or uterine infections. In fact, in only a single case did we find uterine lesions. Of the 20 cows from which it was possible to obtain a record, 14 calved normally and 6 aborted.

It was hoped that by means of some biological tests we would be enabled to recognize infections relatively early. With this in view a number of experiments were undertaken. Though only negative results were obtained, mention of some of the more important ones seems advisable. We attempted to ascertain whether agglutinins for the diphtheroid organism were present in greater concentration in the blood of infected animals than in those known to be free from the disease. The titers were about the same in both groups. Introduction of filtered culture fluid into and beneath the skin and on the cor-

nea failed to result in recognizable reactions. At this time we feel justified in asserting that the only specific diagnosis available consists in the microscopic examination of urinary sediment for the characteristic bacilli.

As already stated several have commented on the appearance of the disease after parturition. Of 19 cases in which statistics were available, 7 were recognized during the first 3 months following parturition, 8 occurred from the 4th to the 6th month thereafter, and 4 from the 7th to the 14th month. It is evident that the disease may occur at any time following parturition. However, statistics of such a nature are liable to mislead since the disease is of long standing in many cases and may not be recognized until the processes are well advanced. On the whole the evidence points to specific infection of the urinary tract independent, in the main, of other abnormalities of the genital tract. This view is strengthened by many bacteriological examinations of the exudates from uterine infections, vaginal secretions, etc. Occasionally diphtheroids of similar character may be met with, but usually only in small numbers.

It is obvious that the specific cystitis and pyelonephritis here described may be of considerable economic importance. It is well recognized as the cause of considerable losses among cows in Europe. Such an infection can become well established within a herd before its presence is known. Although the protocols would appear to show that the actual loss is small it must be borne in mind that many of our cases were slaughtered relatively early in the course of the disease. The record of the small herd within which clinical cases were retained shows the existence of a considerable mortality.

<div align="center">SUMMARY.</div>

A specific infection of the urinary tract associated with a diphtheroid has been encountered in cows from three dairy herds. The most characteristic symptom is the frequent passage of turbid or blood-stained urine. The bladder was always found to be involved. The bladder walls were thickened, the mucosa swollen and reddened. Patches of exudate adherent to the bladder mucosa were frequently observed. In certain instances one, or both ureters were thickened. In other cases one or both kidneys were involved. The kidney proc-

ess seemed to originate in the pelvis and extend toward the medulla and cortex. The characteristic bacilli were always found in large numbers in the urinary sediment and the pelves of involved kidneys.

EXPLANATION OF PLATES.

PLATE 26.

FIG. 1. Bladder of Cow 1152. About ⅔ actual size. The swollen mucous membrane is congested and thrown up into broad folds. Patches of exudate cover portions of the mucosa.

FIG. 2. Bladder of Cow 1153. About ⅔ actual size. The mucosa appears swollen and congested. Several well defined raised patches of exudate are scattered over the mucosa.

FIG. 3. Section of the bladder of Cow 1152. A portion of loosely adherent necrotic epithelium still remains. Two well defined hemorrhages are visible in the submucosa. The disappearance of the mucous membrane permits the passage of red cells into the urine. Engorgement of the capillaries and edema of the submucous connective tissue are pronounced. Stain, eosin and methylene blue. × 86.

PLATE 27.

FIG. 4. Section of bladder of Cow 1153. The section was stained by the Gram method and counterstained with carmine. The black masses beneath the necrotic exudate are masses of bacilli. × 86.

FIG. 5. A higher magnification from the edge of one of the black masses in Fig. 4. Relatively few of the diphtheroids are in focus owing to the thickness and density of the mass. × 810.

FIG. 6. Film from the urinary sediment of Cow 1154. Bacilli are present in considerable numbers. Methylene blue. × 810.

PLATE 28.

FIG. 7. The left kidney of Cow 1154. Most of the cortex appears gray, owing to connective tissue proliferation and round cell infiltration. Note the thickening of the large tubules and the infiltration of the pelvis with connective tissue. About ⅔ actual size.

FIG. 8. Section of the cortex of the left kidney of Cow 1154 stained with eosin and methylene blue. A portion of the original structures has been obliterated by connective tissue proliferation and accumulations of round cells. Well defined periglomerular and peritubular accumulations of round cells are illustrated. Two tubules plugged with leucocytes are also visible. × 86.
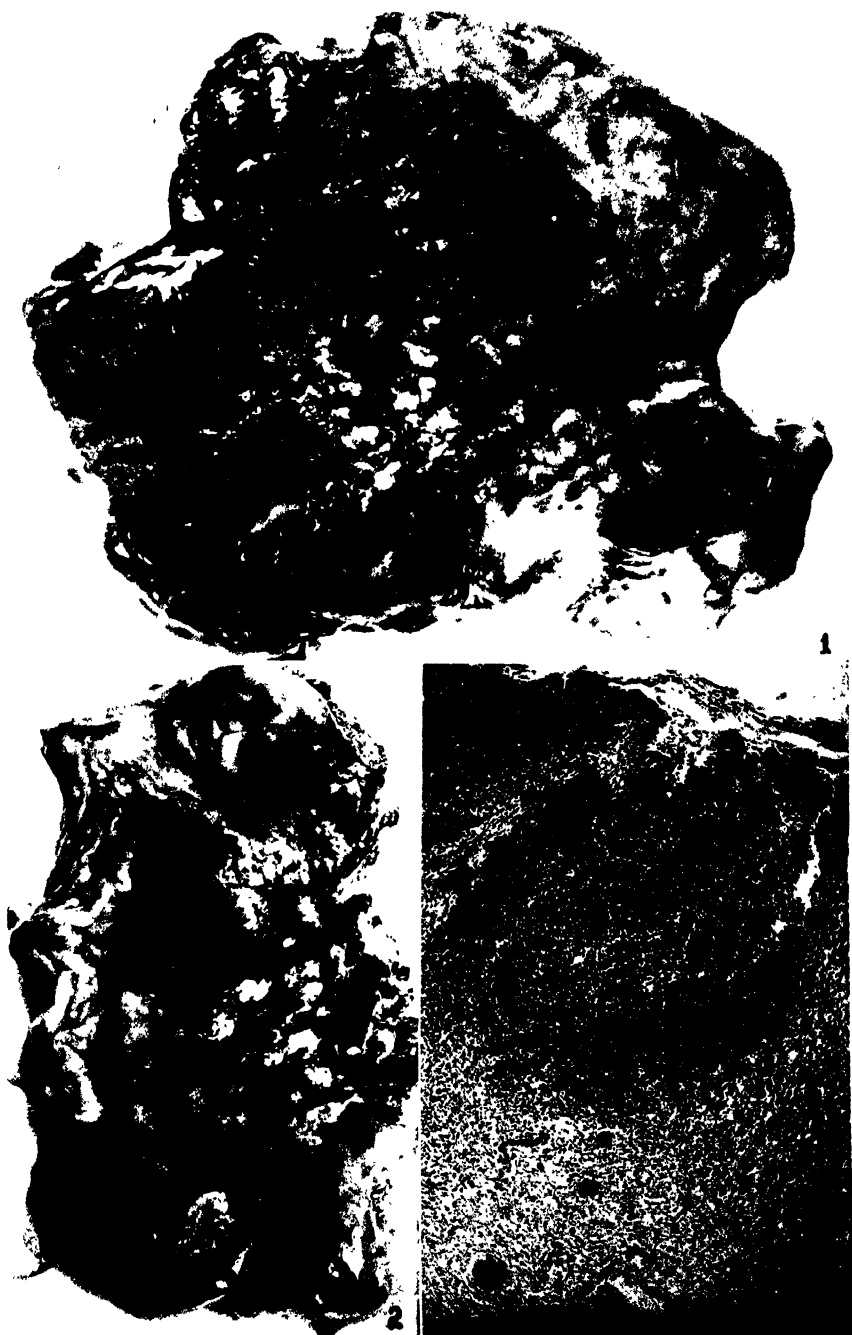
(Jones and Little: Cystitis and pyelonephritis.)
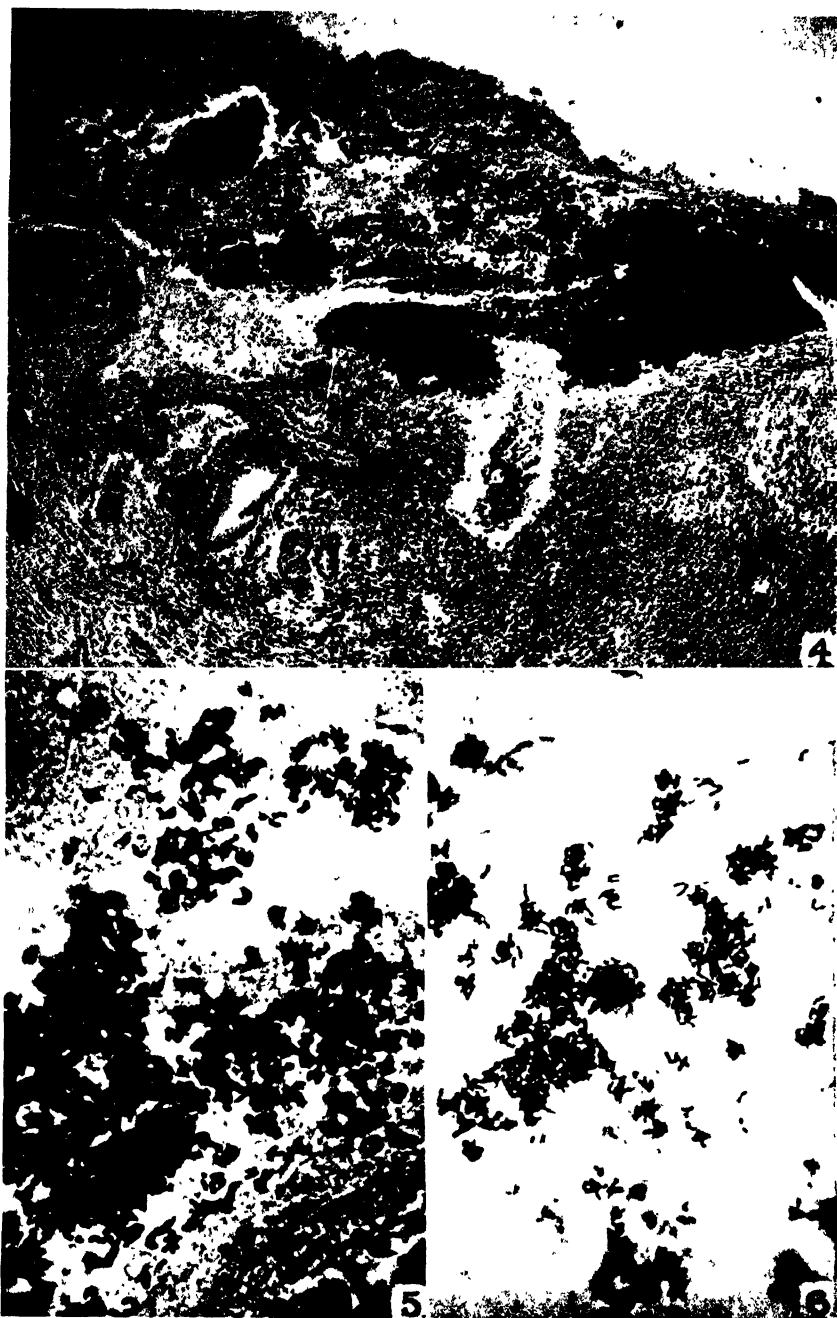
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(Jones and Little: Cystitis and pyelonephritis.)

SPECIFICITY IN BACTERIAL DISEASE WITH SPECIAL REFERENCE TO SILKWORMS AND TENT CATERPILLARS.

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ABSTRACT

The silkworm disease bacillus which is also pathogenic for tent caterpillars produces a specific disease in the two insects when their food is contaminated with the microörganism. This disease cannot be reproduced by contaminating the food with bacteria of general occurrence or with those pathogenic to other insect species. By contaminating the food with other bacteria a few deaths are obtained but these deaths in no way resemble those produced with the silkworm bacillus, and epidemics cannot be initiated. All insects investigated (silkworms, tent caterpillars, army worms, grasshoppers, roaches, etc.) succumb in a few days when inoculated with living cultures. Such a procedure, however, throws little light on the nature and course of a naturally acquired infectious disease in insects.

Beginning with Pasteur in 1870 (1) up to the present time several species of bacteria have been described as the cause of specific diseases in silkworms. Forbes (2), Cohn, Macchiali (3), Paillot (4), Glaser (5). Sawamura (6), on the other hand, in 1906, claimed that he could produce death in silkworms by infecting them with almost any bacterium of general occurrence, and other workers have from time to time drawn inferences from these experiments to explain the bacterial affections of this insect. Sawamura's observations have been verified many times by the present writer. When silkworms, tent caterpillars, or any other lepidopterous forms are inoculated with large doses of any living bacterium they invariably succumb in a few days. An open circulation immediately distributes the organisms which rapidly multiply and overwhelm the host. Such a condition, however, has little in common with the acquisition and development of natural disease. Ingestion of microörganisms with the food is the only manner of bacterial infection so far known in insects and, therefore, it appears

necessary to imitate the natural mode of infection during experimentation.

Silkworms and other herbivorous lepidoptera, when reared on their normal food plants, have scarcely any intestinal flora. In such forms it is often extremely difficult to obtain cultures of bacteria from the gut and nearly always impossible to find bacteria in sections or films prepared from the various parts of the alimentary system. For this reason, as well as for the reasons stated above, it was thought possible that healthy silkworms and other normal herbivorous lepidoptera might be extremely sensitive to almost any bacterial activity occurring within the intestinal tract. In other words, although unlikely, the possibility existed that any bacteria might produce disease in such larvae when conditions within the host became favorable to the bacteria.

The bacillary septicemia in silkworms, described by the writer in 1924, offered the opportunity to compare the behavior of this disease with diseases produced by adding to the food various species of bacteria. Incidentally, the American tent caterpillars were also found to be highly susceptible to the silkworm bacillus and were therefore likewise drawn into the experiments.

A large series of experiments was performed by contaminating the food (mulberry leaves) of silkworms and the food (apple or cherry leaves) of tent caterpillars with seven species of bacteria. The bacteria used were: a variety of *Bacillus coli* derived from the intestine of calves; *Staphylococcus pyogenes aureus*, and *albus; Coccobacillus acridiorum*, varieties *"Souche Cham"* and *"Souche Sidi;"*[1] *Staphylococcus muscae;*[2] and a motile, spore-bearing and proteolytic bacillus which is a common inhabitant of house flies in this vicinity. Each experiment was accompanied by a similar group of silkworms and tent caterpillars infected by adding to their food the silkworm bacillus. Uninfected controls were also held.

Among the uninfected controls none died. The groups of silkworms and tent caterpillars infected with the silkworm bacillus yielded the usual high mortality among the larvae and pupae. (Twenty-five matured out of 160 infected individuals.) These deaths were all

[1] Pathogenic to grasshoppers.
[2] Pathogenic to adult house flies.

typical with the characteristic post-mortem appearances, including the darkening of the skin and complete lysis of all the tissues. Stained films and cultures demonstrated that the silkworm bacillus was the dominant form present in the cadavers; indeed, in many cases it was the only form demonstrable.

The groups of silkworms and tent caterpillars infected with either the colon bacillus, or *Staphylococcus pyogenes aureus, albus, muscae,* or the bacillus from flies all survived and matured. (One hundred infected and 100 moths emerged.)

Out of 15 tent caterpillars and 15 silkworms infected with *Coccobacillus acridiorum*, strain "*Souche Cham*," no tent caterpillars and two silkworms died. Out of 15 tent caterpillars and 15 silkworms infected with *Coccobacillus acridiorum*, strain "*Souche Sidi*," one tent caterpillar and four silkworms died. These seven deaths did not resemble deaths from disease caused by the silkworm microörganism. The skin remained tough and white for several days after death, and the tissues did not liquefy but the worms shriveled and became mummified later. Before death the blood was flooded with the *coccobacilli*. This disease did not assume the form of an epidemic among the experimental animals, in contrast to those infected with the silkworm bacillus, among which one or two deaths occurred each day after the first death.

REFERENCES.

1. PASTEUR, L. M. 1870. Études sur la maladie des vers à soie. La Flacherie, Tome i; pp. 207–317.
2. FORBES, S. A. 1886. Studies on the contagious diseases of insects. Bull Ill. State Lab. Nat. Hist., ii, pp. 257–321.
3. MACCHIALI, L. 1891. Le Stazioni Sperimentale Agrar. Ital. xx.
4. PAILLOT, A. 1922. Les maladies bactériennes des insectes. Ann. des Epiphyties, viii, pp. 95–276.
5. GLASER, R. W. 1924. A bacterial disease of silkworms. Journ. Bacter., ix, pp. 339–352.
6. SAWAMURA, S. 1906. Note on the bacteria pathogenic to silkworms. Bull. Coll. Agri., Tokyo Imp. Univ., vii, p. 1.

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